PERIPHERAL AND CENTRAL ANGIOTENSIN II: PHYSIOLOGIC EFFECTS AND INTERACTIONS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1986

ACKNOWLEDGEMENTS

Sincere appreciation is expressed to my mentor, Dr. Melvin J. Fregly for his enduring patience, guidance, and support. His belief in the fundamentals of physiology and literacy influenced the designing of each project undertaken, and I learned the meaning of scientific integrity, creativity, and serendipity.

I am indebted to the members of my committee, Dr. Neil Rowland, Dr. Ian Phillips, and Dr. Colin Sumners for their encouragement, assistance and productive criticism of my research projects. I am especially grateful to Dr. Sumners who allowed me the privilege of working in his lab and who provided support and advice throughout the duration of this endeavor.

The faculty of the Department of Physiology are also gratefully acknowledged for establishing the physiological background upon which all experiments were and will be designed.

I would also like to thank all the members of the lab for their skillful technical help: Thomas Connors, Howard Clark, Charlotte Edelstein, Jim Reily, Damian Ayers, Dominick Jaeger, Susan Hathaway and John Neil, for all the photographs.

A special thanks is also extended to my parents and friends for their patience, understanding and support.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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May 1986

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Major Department: Physiology

Renin-angiotensin systems (RAS) are present in both the periphery and brain. However, the physiological role of the central RAS and its relationship to the peripheral RAS remain unclear. Initial studies, designed to elucidate the mechanisms through which drinking is elicited, suggest that the dipsogenic responses to angiotensin II (AII) and other dipsogenic stimuli are mediated by an alpha-adrenergic mechanism. A model to support these observations is presented in which two separate pathways, representing osmoreceptor- and AII-mediated drinking, converge on a final common pathway.

The previously undefined role of AII in temperature regulation was also established. Either peripheral or central administration of AII induced a dose-related, hypothermic response manifested by a decrease in heat production and an increase in heat loss mechanisms. The response appeared to be specific for AII and mediated by mechanisms distinct from AII-induced pressor and dipsogenic responses. Administation of AII also affected the mechanisms subserving heat loss and heat production differently: the fall in colonic temperature appears to involve a central cholinergic component, whereas the processes involved in the vasodilation of the tail are unclear but may involve vasopressin.

The relationship between the peripheral and central RAS was further defined by relating changes in circulating AII and mineralocorticoid hormones to the

alterations in both AII receptor binding in the hypothalamus-thalamus-septum (HTS) and the physiological responses to AII. AII receptor binding in the HTS was significantly increased when circulating levels of AII were either elevated with chronic infusions of AII or depressed by treatment with deoxycorticosterone acetate (DOCA). Both drinking and pressor responsiveness to either peripherally or centrally administered AII were elevated in the DOCA-treated rat. In AII-infused rats, increases were observed in daily water intake and urine output which could be significantly correlated with AII receptor binding in the HTS.

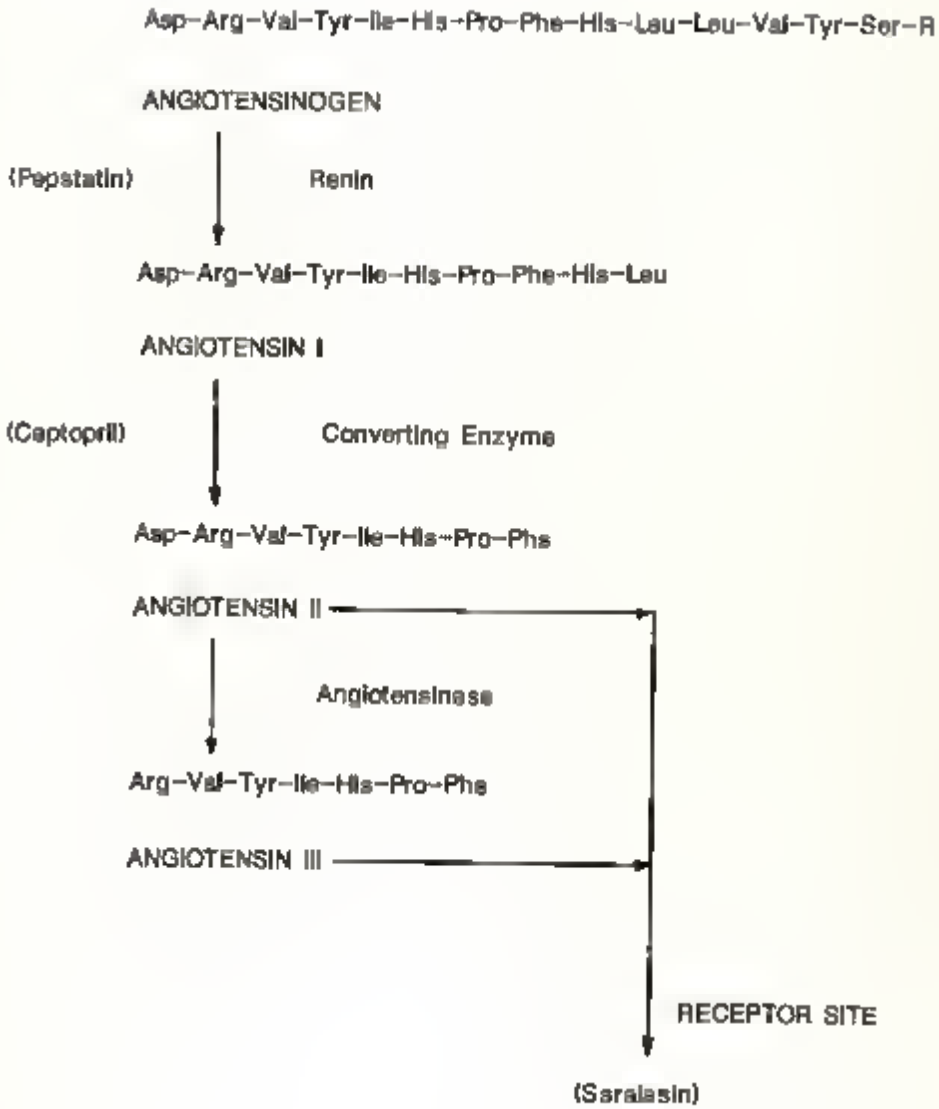
The alterations in AII receptors in the HTS appear to be influenced by elevated plasma concentration of mineralocorticoid hormones rather than AII as suggested by the observations that: (a) no changes were observed in central AII receptors when the increase in aldosterone was prevented by adrenalectomy, and (b) treatment of neuronal brain cell cultures with either DOCA or aldosterone induced a time- and concentration-dependent increase in AII binding to neuronal receptors.

CHAPTER 1
INTRODUCTION

General Introduction

The renin-angiotensin system is well known for its physiological role in cardiovascular, fluid and electrolyte homeostasis. The sequence of events involved in the renin-angiotensin cascade has been well established (Fitzsimons, 1980). In response to either a reduction in blood pressure, increased sympathetic discharge, or a decrease in the total sodium load delivered to the distal tubule of the kidney, the proteolytic enzyme, renin, is released. Renin is synthesized and secreted primarily by the juxtaglomerular cells of the kidney and acts upon the circulating alpha$_2$-globulin, angiotensinogen, which is produced by the liver, to form the decapeptide, angiotensin I (AI). AI is converted to the octapeptide, angiotensin II (AII), primarily in the lung by the converting enzyme. AII is the active component of the renin-angiotensin system and is inactivated by aminopeptidases (angiotensinases) in the blood. One of the breakdown products, the heptapeptide, angiotensin III, also has biological activity which accounts for part of the AII-induced pressor and dipsogenic responses as well as the release of aldosterone (Blair-West et al., 1971; Fitzsimons, 1970; Wright, 1984). A separate renin-angiotensin system has been proposed to exist in the central nervous system (CNS) (Fisher-Perrario et al., 1971; Ganten et al., 1971; Phillips et al., 1979). The evidence and implications of this system will be discussed. A schema of the renin-angiotensin hormonal cascade is presented in Figure 1.

Figure 1. Schematic outline of the renin-angiotensin system. The components identified in parenthesis (to the left of the main schema) are substances known to block either the corresponding enzyme or receptor site identified to the right of the cascade.


Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser-R
ANGIOTENSINOGEN
(Pepstatin)
Renin
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
ANGIOTENSIN I
(Captopril)
Converting Enzyme
Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
ANGIOTENSIN II
Angiotensinase
Arg-Val-Tyr-Ile-His-Pro-Phe
ANGIOTENSIN III
RECEPTOR SITE
(Saralasin)

The primary effects of AII are proposed to be mediated by the interaction of AII with receptor sites in both peripheral tissues and the central nervous system. In the periphery, AII interacts with receptors on arteries to induce vasoconstriction (Regoli et al., 1974) and increase arterial pressure. Receptors in the adrenal cortex are also sensitive to AII which stimulates the release of aldosterone (Davis et al., 1962; Laragh et al., 1960) and consequently induces the retention of sodium by the kidneys. AII additionally modulates synaptic transmission in the sympathetic nervous system by affecting the release (Zimmerman et al., 1972; Hughes and Roth, 1971), synthesis (Roth, 1972), and reuptake mechanisms of neurotransmitters (Davila and Khairallah, 1970). In the CNS, AII stimulates receptors to elicit increases in blood pressure that are distinct from those produced by peripheral vasoconstriction (Bickerton and Buckley, 1961). AII also acts at a central site to induce the ingestion of water (Epstein et al., 1970), the release of vasopressin (Bonjour et al., 1976; Hoffman et al., 1977; Ramsay et al., 1978; Severs et al., 1970) and adrenocorticotrophin (Ramsay et al., 1978) and the stimulation of sodium appetite (Fitzsimons and Striker, 1971; Fregly, 1967). Most of these effects have been elicited by administration of AII into either the cerebrospinal fluid (CSF) or distinct regions of the brain at doses which are ineffective when administered systemically. However, taking into account the volume and accessibility to the sites of action, at equilibrium, the concentration of AII resulting from peripheral administration may not be different from that resulting from central administration.

Historical Background

In 1898, Tigerstedt and Bergman injected saline extracts of kidney into anesthesized rabbits and observed a prolonged rise in arterial pressure. This substance, which was found only in the cortex of the kidney and was more effective in nephrectomized animals, was named renin. Despite early

recognition, the significance of renin was not appreciated until 1934 through experiments by Goldblatt et al. The application of a clip to one renal artery in the dog induced a prolonged rise in blood pressure. The induced-hypertension was postulated to be of renal origin and probably involved renin. Independent studies by Page and Helmer (1940) and Braun-Menendez et al. (1940) established that renin itself was not the pressor substance, but an enzyme. Its effects were produced through the activation of a substrate in plasma to form a pressor substance, originally named angiotonin. This substance was later renamed angiotensin II.

The contribution of the renin-angiotensin system to fluid balance was recognized in 1960 with the discovery that AII was an important regulator of the release of aldosterone (Laragh et al., 1960). Aldosterone directly stimulates the reabsorption of sodium from the distal tubules and collecting duct of the kidney. The observation that the same hormonal system might be responsible for the initiation of drinking did not become apparent until the investigations by Fitzsimons in 1964. In this study, nephrectomized rats were less willing than normal rats to drink when the inferior vena cava was ligated. It was proposed that the kidney played a role in the drinking induced by ligation of the vena cava by releasing a renal factor, possibly renin. In subsequent studies, bilateral nephrectomy was shown to reduce drinking to some hypovolemic stimuli (Fitzsimons, 1966). In 1969, Fitszimons demonstrated that intravenous injections of either renin or angiotensin II to the rat elicited drinking. Since then, AII has been observed as a potent dipsogen in a number of species of animals including dog, cat, goat, birds, eel and iguana (Fitzsimons, 1980).

The first indication that AII may have an effect mediated through the central nervous system was demonstrated in a cross-circulation experiment conducted by Bickerton and Buckley (1961). The head of one dog was

disconnected from its peripheral vasculature and reconnected to the jugular and carotid veins of another dog. An intracarotid injection of AII into the intact dog induced an increase in the pressure of the connected dog. This pressor response was interpreted to be independent of the vasocontrictor action of AII on vascular smooth muscle and mediated, instead, by the elevation of cerebral angiotensin which increased sympathetic outflow through the spinal cord. In 1967, Severs et al. administered low doses of AII directly into the brain and induced a pressor response. Infusions of AII into the vertebral artery of the dog were also shown to elicit an increase in blood pressure at doses which were not effective with either intracarotid or intravenous infusions (Ferrario et al., 1972; Scroop and Lowe, 1969). In rats, however, intracarotid infusions, but not intravenous or intravertebral administration, elicited the pressor response, suggesting differences between species (Haywood et al., 1980). Many of the effects of circulating AII are now known to occur through an interaction with its specific receptors in the brain.

AII-Induced Drinking and Water Balance

In his classical experiments of regulatory physiology, Adolph demonstrated the importance of water intake to the maintenance of body fluid homeostasis (1939). Two separate pathways which involve the depletion of fluids from either intracellular or extracellular compartments have been proposed to mediate drinking. Extracellular fluid volume (ECF) can be altered by dehydration, hemorrhage, and the administration of either hypertonic saline or polyethylene glycol, and drinking is induced. Administration of hypertonic saline influences intracellular fluid (ICF) volume by affecting ECF osmolality and initiates drinking via an osmoreceptor pathway (Andersson, 1977; Greenleaf and Fregly, 1932). Drinking induced by isoproterenol, aortic constriction above the renal arteries, caval ligation, and constriction of the renal arteries are either reduced or abolished by nephrectomy, while that

accompanying hypovolemia induced by hyperoncotic colloid is not affected (Fitzsimons, 1969; Houpt and Epstein, 1971). Each of these stimuli involves the release of renin from the kidneys. These investigations led to the hypothesis that water intake induced by changes in ICF were mediated by osmoreceptors, and drinking elicited by ECF volume depletions involved the renin-angiotensin system. However, some drinking stimuli, such as denydration and PEG, are not totally abolished by agents which block either the release of renin or other components of the renin-angiotensin system, which suggests that some forms of drinking may be mediated either by both pathways (Fitzsimons, 1980; Greenleaf and Fregly, 1982) or by an additional volumetric pathway (Striker, 1968). The observation that intravenous (i.v.) administration of either renin or AII (Fitzsimons, 1969) could initiate drinking, and subsequent studies which demonstrated that intracerebroventricular (i.v.t.) administration of angiotensinogen, renin, AI and AII also elicited drinking at doses which were not effective when administered systemically, (Epstein et al., 1970; Fitzsimons, 1971; Severs et al., 1970) completed the schema of the physiological role of AII in drinking. Either hypotension, hypovolemia, or a sodium load stimulates release from the kidney of renin which reacts with circulating angiotensinogen to form AI. AI is converted to AII which stimulates receptors in the brain to elicit thirst. The threshold plasma concentration of AII to induced drinking has been estimated at 125-200 fmol of AII/ml by various investigators (Epstein, 1978; Mann et al., 1980a; Simpson et al., 1978). Thus, stimulation of the endogenous renin-angiotensin system by stimuli such as dehydration, generates levels of AII exceeding the dipsogenic threshold (Mann et al., 1980a) and gives further support for the physiological role of the endogenous renin-angiotensin system.

Much debate exists as to which area of the brain mediates thirst elicited by AII. The subfornical organ (SFO) (Simpson and Routtenberg, 1973), organum

vasculosum of the lamina terminalis (OVLT) (Hoffman and Phillips, 1976a), and the anteroventral third ventricular (AV3V) (Buggy and Fisher, 1976; Hoffman and Phillips, 1976b) regions in the brain have all been implicated because doses of AII as low as 10^{-16} to 10^{-15} mol. can induce water intake, and independent ablation of each area affects water intake accompanying either central or peripheral administration of AII. Present evidence suggests that the OVLT mediates drinking to both peripheral and i.v.t. routes of administration, whereas the SFO appears most accessible to blood borne angiotensin, and receptors in the AV3V region are directly stimulated by centrally administered AII. The AV3V region, however, appears to be necessary for drinking elicited by both i.v.t. and systemic administration. Disruption of prominent neural projections between the SFO and the AV3V region inhibits drinking to peripherally, but not centrally administered AII (Lind and Johnson, 1982). Lesions of the AV3V region, on the other hand, block the drinking to both routes of administration (Johnson and Buggy, 1978). Specific areas of the AV3V region include the nucleus medianus (Nelson and Johnson, 1985) and the median preoptic nucleus (Lind and Johnson, 1982; Shrager and Johnson, 1977).

Several different mechanisms have been suggested for the dipsogenic effect of AII (Fitzsimons, 1980). Felix and Schlegel (1978) have shown increased firing activity of neurons in the SFO accompanying direct application of AII. AII has also been demonstrated to modulate the presynaptic release of neurotransmitters involved in drinking, in particular, acetylcholine and norepinephrine. Cholinergic blockade has been shown to inhibit AII-induced drinking, but since the doses necessary were much larger than those needed to block cholinergic drinking, the physiological significance of this inhibition remains questionable (Fitzsimons, 1980; Severs et al., 1967). The participation of catecholamines, on the other hand, is much more conclusive. AII is postulated to influence the synthesis, release and reuptake of catecholamines

involved in the pressor and dipsogenic responses induced by AII (Fuxe et al., 1979; Gordon et al., 1979; Summers and Phillips, 1983). Dopaminergic, but not B-adrenergic, receptors have been implicated (Fitzsimons and Setler, 1971). The effect of alpha-adrenoceptor blockade has been controversial, but most recent evidence indicates the participation of central $alpha_2$-adrenoceptors (Fregly et al., 1983a; Fregly et al., 1984a,b).

In addition to the initiation of drinking, AII also contributes to water balance by stimulating the release of aldosterone and antidiuretic hormone (ADH); by direct effects upon glomerular filtration and tubular reabsorption, and by its participation in sodium appetite (Fitzsimons, 1980; Reid et al., 1978). The renin-angiotensin-aldosterone axis is of particular interest, because increased levels of aldosterone will feed back to deplete directly the kidney of renin (Tobian, 1959). Treatment with the aldosterone precursor, deoxycorticosterone acetate (DOCA), has also been demonstrated to alter AII-mediated responses and these effects may occur at the level of the AII receptor (Douglas and Brown, 1982; Schiffrin et al., 1983). Whether these effects are either direct or a manifestation of decreased levels of AII is unknown.

Receptor Regulation

The notion that drugs interact with a receptor substance to produce a response was introduced by Langley (1878), and elaborated by Ehrlich (1900, 1909), who proposed that molecules of drugs interact with chemically reactive groups (receptors) to produce a biological response. The physiological effects of most peptides and hormones are now known to be mediated by their interaction with membrane-bound receptors. The stimulation of target cells by hormones initiates a complex series of events, which in addition to affecting a cellular response, also regulates the components of the effector-receptor complex and modifies subsequent responses to stimulation. Intrinsic to the regulation of

membrane-bound receptors is the concept of the fluid mosiac model of membrane structure (Singer and Nicholson, 1972) and the implication that the mobility of receptors in the membrane underlies receptor activation and regulation (Swillens et al., 1977). Target cells have been shown to respond to changes in ligand concentration by regulating either the number or affinity of the surface receptors. Increased hormone concentration typically induces a decrease in the respective receptors (Catt et al., 1979). This self-regulation of membrane receptors has been observed with several ligands including peptides, neurotransmitters, protein-hormones, and by surface modulating factors, lectins and immunoglobulins (Raff, 1976). However, there are exceptions to this generalization of which AII receptors are a prime example. In the presence of high concentration of the ligand, angiotensin II, an increase in the number of adrenal AII receptors is observed (Hauger et al., 1978). In addition, many peptide hormones are regulated by other hormones.

Implicit in the regulation of receptors, is the expectation that end-organ responsiveness will also be changed by alterations in either the number or affinity of receptors. Indeed, desensitization of cellular responses by increased ligand concentration has been documented, and in certain target cells this alteration has been correlated with a reduction in receptor number (Catt et al., 1979). Conversely, in the adrenal gland, an increase in AII receptors is observed during elevated circulating levels of AII and this correlates with an increase in AII-induced aldosterone release (Aguilera et al., 1978; Hauger et al., 1978).

Several studies on the binding of AII to its receptors have been conducted to determine the molecular nature for the physiological actions of AII. Specific receptor sites for angiotensin II have been identified and characterized in the adrenal zona glomerulosa (Glossman et al., 1979; Hauger et al., 1978), kidney (Brown et al., 1980; Mendelsohn et al., 1983), vascular

smooth muscle of the uterus (Devynck et al., 1976), bladder (Catt et al., 1984), and arteries (Gunther et al., 1980), aorta (Le Movan and Palaic, 1975), anterior pituitary (Hauger et al., 1982), and the brain (Mann et al., 1980b; Sirett et al., 1977). The binding sites are characterized by high specificity and high affinity for the peptide. All binding sites also have been analyzed during cell fractionation and are predominantly localized in the plasma membrane (Catt, 1984). In the brain, specific (^{125}I)-AII binding sites are localized to the midbrain, septum, hypothelamus, thalamus, medulla, and olfactory bulb. Specific binding of AII in the cortex, hippocampus, striatum, and cerebellum is extremely low (Sirett et al., 1982). The localization of AII receptors in the brain correlates with the areas of the brain which mediate several of the physiological effects of AII. The central region of the brain which contains the hypothalamus, septum, thalamus and midbrain, is often used by investigators to characterize AII receptors (Mann et al., 1980b; Mann et al., 1981; Sirett et al., 1982). The binding of AII to receptors in the brain has been found to be of high affinity and specificity, reversible, saturable and pH- and temperature-dependent. The specificity of the receptors for several synthetic AII-analogues is similar in the brain and adrenal (Douglas et al., 1980; Mann et al., 1981). The competition for binding sites and potency of AII peptide fragments and antagonists in vitro correlates with potency in vivo (Mann et al., 1981). The equilibrium dissociation constant (K_d) obtained in these studies was 0.2-1.0 nM and the maximal binding capacity (B_{max}) was estimated at 11-20 fmol/mg protein. Other investigators have reported similar values (Bennet and Synder, 1976; Cole, 1980). Angiotensin receptors in the brain have been visualized with fluorescent microscopy (Landas et al., 1980) and quantified through autoradiography (Israel, 1984; Healy and Printz, 1985). Specific AII receptor binding has also been identified in neuronal brain cell cultures (Raizada et al., 1981; Printz et al., 1984) and exhibits similar

kinetics as observed in membrane preparations from the brain of the adult rat.

Angiotensin II receptors have been shown to be regulated by endogenous hormone concentration as discussed above. However, the receptors are not regulated similarily in all tissues and are sensitive to electrolyte concentrations in the blood. The regulation of AII receptors in smooth muscle is opposite to that seen in the adrenal gland. Elevated levels of plasma AII tend to induce desensitization of both smooth muscle receptors and responsiveness (Aguilera and Catt, 1981; Gunther et al., 1980; Mann et al., 1981). The extent to which circulating AII regulates receptors in the central nervous system is presently unclear. The question remains as to whether AII can cross the blood-brain barrier to regulate central AII receptors.

Angiotensin II and the Central Site of Action

The hypothesis that many of the responses elicited by the renin-angiotensin system occur through the central nervous system presents a paradox, because the polar nature of the AII-peptide infers its inability to cross the blood-brain barrier (BBB). The BBB is comprised of capillary endothelia which possess tight junctions between adjacent endothelial cells to limit the entry of molecules on the basis of their lipid solubility and molecular weight (Brightmann and Reese, 1969; Reese and Karnovosky, 1967). However, there are small areas of the brain which lack a blood-brain barrier due to the presence of fenestrated capillaries, or gaps in the capillary endothelium surrounding the brain. These regions are known as the circumventricular organs (CVO) and include the area postrema (AP), subfornical organ (SFO), median eminance (ME), organum vasculosum of the lamina terminalis (OVLT), and the neurohypophysis (Weindl, 1973). Low-resistance tight junctions also exist on the ventricular side of the CVO and form a blood-CSF-barrier to limit the diffusion of solutes past the CVO (Pardridge et al., 1981).

Physiological and immunohistochemical evidence suggest that circulating AII may cross the BBB (Johnson and Epstein, 1975; Volicer and Lowe, 1971). However, large doses of AII were used, and it is suggested that acute increases in blood pressure damage the integrity of the BBB and allowed passage of substances into the brain (Johansson et al., 1970; Phillips, 1980; Westergaard et al., 1977). Other evidence includes administration of the AII-receptor antagonist, saralasin, to block the physiological responses to either centrally or peripherally administered AII. Intracerebroventricular administration of saralasin can block the dipsogenic effect of i.v. administered AII (Johnson and Schwob, 1975). Conversely, saralasin given i.v. can also block the dipsogenic and pressor responses accompanying i.v.t. administration of AII (Johnson and Schwob, 1975, Hoffman and Phillips, 1976a). Additional studies with radiolabeled AII indicated that systemic administration of [^{125}I]-AII (non-pressor dose, 36 pMol) bound only to AII receptors in the circumventricular organs and the hypophysis, but not to any of the other AII receptors located within the BBB (Van Houten et al., 1980). However, in a subsequent study, central administration of saralasin blocked the binding of [^{125}I]-AII to the ME, OVLT, AP, most of the SFO, except the central core, and only a small portion of the adenohypophysis. Intravenously administered saralasin inhibited binding in the same areas as well as the center of the SFO and both lobes of the pituitary (Van Houten et al., 1983). Thus, it remains to be elucidated whether and how AII penetrates the BBB.

The hypothesis of an endogenous renin-angiotensin system in the brain has been advanced to explain actions observed with central administration of AII and the localization of AII binding sites within the BBB (Fisher-Ferrario et al., 1971; Ganten et al., 1971; Phillips, 1978). Several responses are only produced by administration of central, and not peripheral, AII. Selective alterations of brain catecholamines are generated by increases in central

levels of AII (Fuxe et al., 1979). In addition, central administration of AII inhibits prolactin release (Steele et al., 1982), whereas systemic administration actually stimulates the release of prolactin from the pituitary (Aguilera et al., 1981). Each of the components necessary for the generation of AII has been localized by either biochemical or histochemical techniques. The highest concentrations of renin are found in the choroid plexus, anterior pituitary, and the pineal gland (Inagami et al., 1980). Renin has been shown to coexist with AII in certain neuronal cells which suggests that formation of angiotensin may be an intracellular mechanism in the CNS (Inagami et al., 1982). The other enzyme necessary for the generation of AII, converting enzyme, has been identified in capillary endothelial cells of the brain, the SFO and the choroid plexus (Reid et al., 1982; Rix et al., 1981). Angiotensinogen has also been found throughout the CNS and is in particlarly high concentrations in the CSF (Lewicki et al., 1978; Schelling, 1983). AII has been localized by radioimmunoassay and immunocytochemistry in the hypothalamus, spinal cord, medulla oblongata and the limbic system. The highest densities of AI and AII were found in the periventricular structures, SFO and the ME (Changaris et al., 1977; Ganten et al., 1978). AI and AII have also been extracted from the brain, characterized by high pressure liquid chromatography (HPLC) and generated during incubations of brain-angiotensinogen with renin (Hermann et al., 1984; Phillips and Stenstrom, 1985).

Substantial evidence has been presented for an endogenous renin-angiotensin system in neuronal brain cell cultures separate from circulating AII. Angiotensinogen (Printz et al., 1984), AII (Raizada et al., 1984b), and AII receptors (Printz et al., 1984; Raizada et al., 1981) have been identified in neuronal cell cultures prepared from the brains of one-day-old rats. AII-like immunoreactivity extracted from neuronal cultures has been shown to co-migrate with synthetic AII (Raizada et al., 1984b). In addition, the neuronal cultures

were able to synthesize immunoprecipitable [^{3}H]-AII from [^{3}H]-isoleucine and [^{3}H]-valine (Raizada et al., 1983). The kinetics for binding of [^{125}I]-AII to brain cells in culture were similar to those observed in membranes from adult rats. The binding was specific, saturable, pH-dependent, and reversible. Autoradiographic studies also indicated that the binding of AII was localized to neurites and neuronal cell bodies (Raizada et al., 1981). Weyhenmeyer and colleagues (1984) have extracted angiotensin from cultures prepared from the brains of fetal rats, and induced drinking in rats which received the extract. Thus, the identification, synthesis and bioactivity of angiotensin in neuronal brain cell cultures further supports the existence of an endogenous brain renin-angiotensin system.

Specific Aims

Although all of the components of the renin-angiotensin-system have been identified in the brain, its physiological role has not been defined. The localization of receptors for AII on the circumventricular organs obviates a need to pass beyond the blood brain barrier to elicit a response. Yet, administration of AII directly into the CNS activates receptors which cannot be activated by circulating AII. Furthermore, the factors influencing the regulation of the renin-angiotensin system in the brain have not been elucidated.

Evidence suggests that chronic administration of DOCA can reduce plasma levels of AII by actions directly on the kidney to reduce renin release (Tobian, 1959). Alternatively, chronic infusions of low doses of AII increase plasma AII levels. Both of these effects have been demonstrated to alter AII receptor binding capacity in peripheral tissues in opposite directions (Douglas and Brown, 1982; Schiffrin et al., 1983, Schuffrin et al.,1984). However, the effect of chronic treatments with DOCA and AII on the regulation of AII receptors in the brain has not been investigated. Either the presence or

absence of an effect of experimentally induced alterations in plasma concentrations of AII and mineralocoticoid hormone on AII receptor binding capacity in the brain could contribute to an understanding of the interrelationship between the renal and putative brain renin-angiotensin systems.

Although investigations at the level of the receptor will serve to explain the biochemical link of the two systems, the potential physiological role can only be evaluated through the effect that these treatments have on the biological responses. Thus, studies were designed to elucidate further the physiological effects of peripherally and centrally administered AII, the mechanisms underlying these responses, and resulting consequences of alterations in central AII receptors.

CHAPTER II
GENERAL METHODS

Treatment regimens and descriptions pertinent to separate studies will be detailed in each experiment. However, several of the methods are common between experiments and will be described here. Male and female rats of the Blue Spruce Farms (Sprague-Dawley) strain weighing 200-400 g were used in the drinking, blood pressure, temperature and AII membrane binding experiments described here. They were maintained in groups of three to four in stock cages and allowed tap water and Purina Laboratory Chow ad libitum. The colony room and the adjacent quiet testing room were maintained at 26 $\pm$ 1° and illuminated from 0700 to 1900 hours (hr). Measurements were made at the same time each day (0900 hr) to minimize any potential effects of circadian rhythm. For experiments with cell culture, one day old rats were obtained from our breeding stock of Sprague-Dawley (Blue Spruce Farms) rats. All drugs and chemicals used in the experiments are listed in the Appendix.

Measurement of Drinking Responses

Two types of drinking responses were evaluated; an acute response to administration of a dipsogen, and a chronic response which considered the daily intake of fluids and its relation to urine output and food intake. For these chronic measurements, the consumption of tap water (or saline when specified) and ground Purina Laboratory rat chow were calculated each day by the loss of weight from the containers. The food containers are spill-resistant and have been described previously in detail (Fregly, 1960). Water containers consisted of infant nursing bottles with cast aluminum spouts as described by Lazarow

(1954). Urine was collected into graduated cylinders which contained small amounts of paraffin oil to prevent evaporation.

For acute dipsogenic tests, each rat was injected with either the appropriate drug or vehicle and then placed into an individual stainless steel cage. No food was available during the experiment. Immediately after the injection, each rat was given a preweighed bottle of water. Water (26°C) intakes were measured gravimetrically at 0.5, 1, and 2 hr after administration of dipsogen.

To test their drinking responses to central administration of AII, rats were implanted with intracerebroventricular cannulae as described below. For this purpose, an injector cannula was attached to a 10 ul Hamilton syringe by PE-10 tubing, and filled with AII dissolved in sterile isotonic saline. Each rat was injected with 10 ng of AII in a volume of 2 ul. Water intake was measured as described above.

All experiments were set up according to a statistical factorial design to assess the effects of treatment and interaction (Snedecor and Cochran, 1956).

Intracerebroventricular Cannulation

To test drinking, pressor and temperature responses to central administration of AII, rats were surgically prepared with indwelling intracerebroventricular cannulae. Each rat was anesthetized with ketamine (130 mg/kg, i.m.) and acepromazine (50 ug/kg, i.m.) and a 12-mm long, 22-gauge stainless steel tube was implanted into the lateral ventricle at least one week before testing. The coordinates were 1.0 mm posterior, 1.0 mm lateral and 5.0 mm deep (from dura) with respect to bregma (flat skull) using a Kopf stereotaxic instrument. The cannula was secured with skull screws and dental cement, and a stainless steel obturator (12 mm long) was placed into the cannula. Each rat received 0.2 mg ampicillin i.m. following surgery, and was allowed one week for recovery.

Measurement of Temperature Responses

To measure the changes in the thermoregulatory responses during experimentation, tail skin and colonic temperatures were measured at an ambient temperature of 26 ± 1°C while the rats were restrained in plexiglass tunnel-type cages. Colonic temperature (CT) was measured with a copper-constantan thermocouple inserted 5 cm into the colon of each rat. An additional thermocouple was placed on the dorsal surface at the base of the tail for the measurement of tail skin temperature (TST). Both thermocouples were secured to the tail with adhesive tape. Temperatures were recorded by a potentiometer at 6 min intervals. Rats were allowed one hr to adjust to the restraining cages and control measurements were recorded for 30 min.

Although the protocols were similar, different drugs were administered in each of the experiments. Rats were separated randomly into equal groups (n=6), and after the initial 30 min control period, each rat was administered either one or two injections. In the experiments in which two drugs were administered, the control group received two subcutaneous (s.c.) injections of the saline vehicle used to dissolve the various drugs (1 ml 0.9% saline/kg). The second and third groups received one drug and the saline vehicle, while the last group received a combination of two drugs. The treatments were given either simultaneously or separated by a specific time interval, depending on the compounds used. The response to the treatment was measured for 120 min. Statistical analysis was carried out by one-way analysis of variance (Snedecor and Cochran, 1956). Comparison between means was made by Student's t-test using the pooled variance from the analysis of variance (Huntsburger, 1961). Significance was set at the 95% confidence level.

Receptor-Binding Studies in the Hypothalamus-Thalamus-Septum

The AII-receptor binding assay and the dissection of the brain of the rat were a modified version of that of Sirett et al. (1977). The brain was cut anterior to the preoptic region and at the level of the mammillary bodies to represent the anterior and posterior limits of the diencephalic block. The lateral limits included the edges of the lateral hypothalamus. The block of tissue (mean weight = 100mg) included the thalamus, hypothalamus, and septum (HTS). Specific areas of this hypothalamic block included the preoptic area, paraventricular nucleus, organum vasculosum of the lamina terminalis (OVLT), subfornical organ (SFO), anterior and posterior nuclei, and dorsomedial and ventromedial nuclei. Following dissection, this block of tissue was homogenized in 20 volumes of ice cold 0.9% saline, and the homogenate was centrifuged at 600 x g and 4°C for 10 min to remove all blood vessels and connective tissue. The supernatants were decanted into another tube on ice and then centrifuged at 50,000 x g at 4°C for 30 min. This supernatant was discarded and the pellet resuspended in 2 ml buffer containing 150 mM NaCl, 5 mM EDTA and 50 mM Tris HCl at pH 7.2 and 4°C. Portions of this particulate fraction (100 ul containing 100-150 ug protein) were placed into microfuge tubes. Half of these tubes were incubated for 30 min at 22°C with 200 ul assay buffer containing 150 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, 50 mM Tris HCl, 0.2% bovine serum albumin and 0.1 to 1.0 nM [^{125}I]-AII (specific activity 1500-1800 uCi/ug). Non-specific binding was determined in the other half of the tubes in parallel by the addition of 10,000 fold excess of unlabeled AII to the reaction buffer described above. All reactions were performed in triplicate and terminated by placing the tubes in ice and adding 1.0 ml ice cold Tris HCl buffer. Separation of bound and free radioactive AII was accomplished by centrifugation at 13,000 x g for 5 min in a microfuge. The membranes were pelleted, and the supernatant was aspirated. The pellet was washed with an

additional 1.0 ml of ice cold Tris HCl buffer and centrifuged for an additional 5 min. The supernatant was aspirated and the tip of the microfuge tube, which contained the membrane pellet, was cut and placed in another tube and the amount of radioactivity was counted in a Beckman 5500 Gamma Radiation Counter, with a counting efficiency of 75% for ^{125}I. The results are expressed as specific binding which was obtained by subtracting non-specific binding from total counts. The specific binding of [^{125}I]-AII for the diencephalic block of tissue used in these experiments was 75-80% of the total counts bound. Protein concentration of the brain particulate fraction was determined by the Lowry et al. (1951), and results were expressed as fmol/mg protein.

CHAPTER III
ANGIOTENSIN II-INDUCED DRINKING: A FINAL COMMON PATHWAY?

Introduction

Studies from a number of laboratories are in general agreement that naloxone, an opioid antagonist, can inhibit the response to a variety of dipsogenic stimuli (Brown and Holtzman, 1981b; Cooper, 1980; Rowland, 1981). These results suggest that opioid receptors may play a role in mediating drinking. Recent studies from this laboratory revealed that clonidine, an alpha$_2$-adrenoceptor agonist, can also inhibit the response to the same dipsogenic stimuli (Fregly and Kelleher, 1980; Fregly et al., 1981). These results suggest that alpha$_2$-adrenoceptors may play a role in mediating drinking. It is of interest that both naloxone and clonidine attenuate the drinking response to dipsogenic stimuli that are believed to act both via osmoreceptors (e.g., hypertonic saline) and AII receptors (e.g., AII). It is therefore likely that both may act at the point of convergence of pathways mediatated by these two receptors. Oatley (1973), Toates (1979), and Greenleaf and Fregly (1982) have proposed that the point of convergence occurs through a summing device, since any two submaximal dipsogenic stimuli applied to the rat result in a summation of the response rather than an interaction. The summing device may be present within the central nervous system but its exact location is not known with certainty. Since the antidipsogenic effects of naloxone and clonidine were similar, an attempt was made to determine whether they might act via similar mechanisms. To this end, studies were carried out to assess the possibility that naloxone might induce its antidipsogenic effect by acting at alpha$_2$-adrenoceptors.

The effects of naloxone and clonidine, separately and in combination, on experimentally-induced drinking will be briefly reviewed; and then new data will be presented which suggest that these two agents may act via a common pathway.

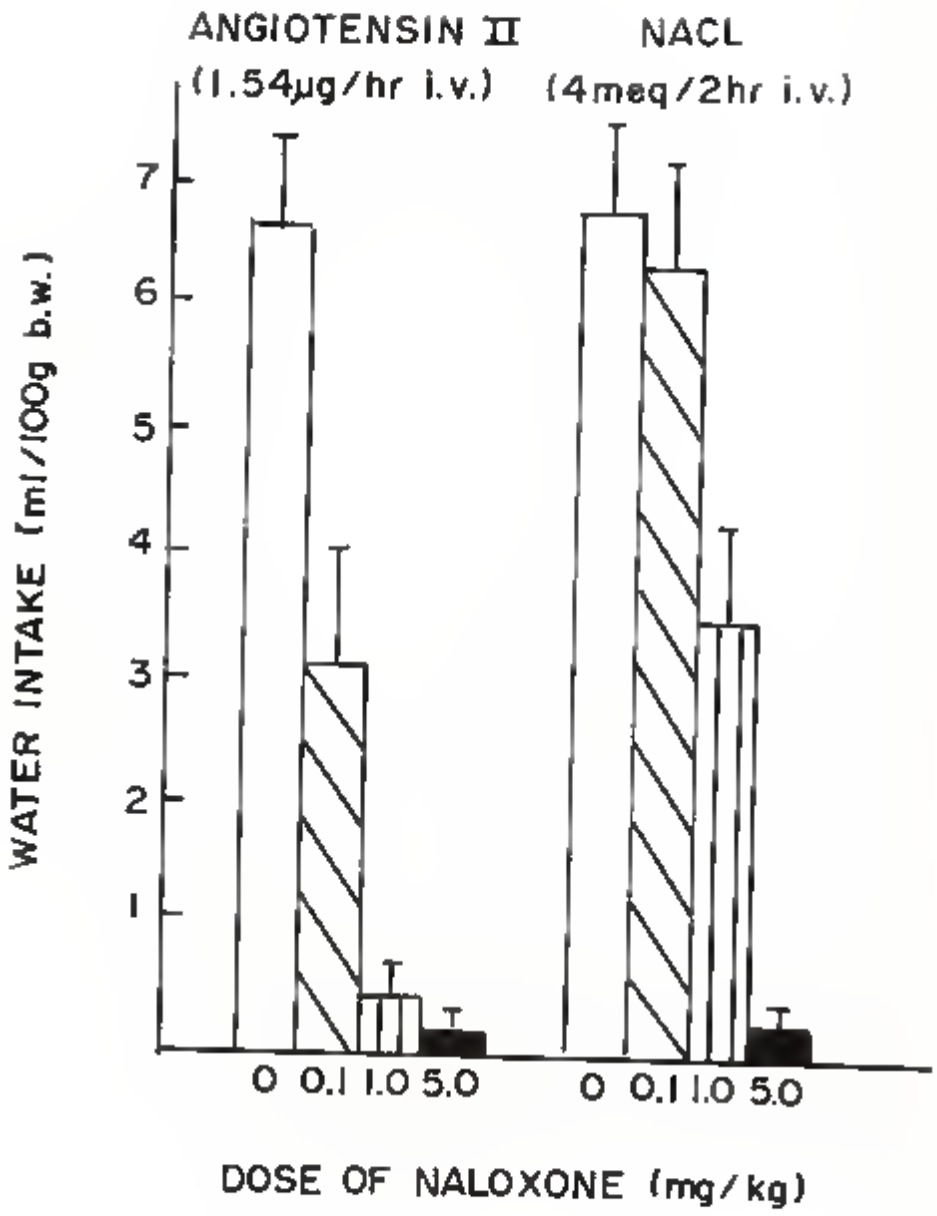
Effect of Naloxone on Experimentally-Induced Drinking

Administration of naloxone produced a dose-related attenuation of the water intake induced by all dipsogens. The magnitude of the effect depended upon the type of stimulus, the route of administration, and the species (Sanger, 1981). This has been most fully documented for dehydration-induced drinking (e.g., Cooper, 1980; Frenk and Rogers, 1979; Holtzman, 1979). However, because fluid deprivation induces both osmotic and AII components of drinking (Ramsay et al., 1977), subsequent analysis of the effects of naloxone on each of these components was made. Thus, the dipsogenic responses induced in rats either by s.c. polyethylene glycol, by i.v. NaCl or AII (Rowland, 1982) or by s.c. isoproterenol (Brown and Holtzman, 1981b) were attenuated by naloxone (see Fig. 2). Further, since quaternary opioid antagonists, which do not cross the blood brain barrier, were without effect on drinking when given peripherally (Brown and Holtzman, 1981; Ostrowski et al., 1981), a central site of action was proposed. Indeed, central administration of either naloxone (Czech et al., 1983) or methyl naloxone (Brown and Holtzman, 1981a) was antidipsogenic.

Effects of Clonidine on Experimentally-Induced Drinking

Administration of clonidine produced a dose-related inhibition of all types of experimentally-induced drinking thus far studied, including that induced by water deprivation, administration of isoproterenol (Fregly and Kelleher, 1980), hypertonic saline, AII, pilocarpine (Fregly et al., 1981), 5-hydroxytryptophan and serotonin (Threatte et al., 1981). An example of its effect on angiotensin II- and isoproterenol-induced drinking is shown in Figure

Figure 2. Antagonism of drinking responses by naloxone, based on data from Rowland, 1982. Male Sprague-Dawley rats with indwelling venous catheters received either $Ileu^5$-AII at 1.54 ug/hr for 1 hr (left panel), or 4 mEq of 2M NaCl over a 2 hr period (right panel). One standard error (SE) is set off at each mean.


ANGIOTENSIN II
(1.54μg/hr i.v.)
NACL
(4meq/2hr i.v.)
WATER INTAKE (ml/100g b.w.)
7
6
5
4
3
2
1
0 0.1 1.0 5.0
0 0.1 1.0 5.0
DOSE OF NALOXONE (mg/kg)

3 (Fregly and Kelleher, 1980; Fregly et al., 1981). It has also been proposed that clonidine acts via the central nervous system because: (a) drinking induced by low doses of angiotensin II administered into the lateral cerebral ventricle was suppressed by peripheral clonidine, and (b) i.v.t. administration of clonidine attenuated the drinking induced by either central or peripheral injections of AII (Fig. 4) (Fregly et al., 1984). However, like naloxone, relatively large doses of clonidine were needed for a central effect. While the antidipsogenic effect of naloxone and clonidine may be centrally mediated, the i.v.t. route of administration may not afford ready accessibility to the site of action (Czech et al., 1983). At the doses used, clonidine had no effect on food intake, running activity (Fregly and Greenleaf, 1981) or drinking of sweet milk (Rowland, unpublished data). Therefore, the antidipsogenic effect of clonidine appears to be relatively specific.

Naloxone and clonidine thus share some common characteristics. Both induce dose-related attenuation of experimental drinking; both appear to be mediated at a central site which is poorly accessible to the CSF, and unlike other antidipsogens such as neuroleptics, their effects on drinking do not seem to be secondary to general sedative or debilitating actions. In view of these parallels, it seemed of some interest to examine clonidine and naloxone for potential common mechanisms of action.

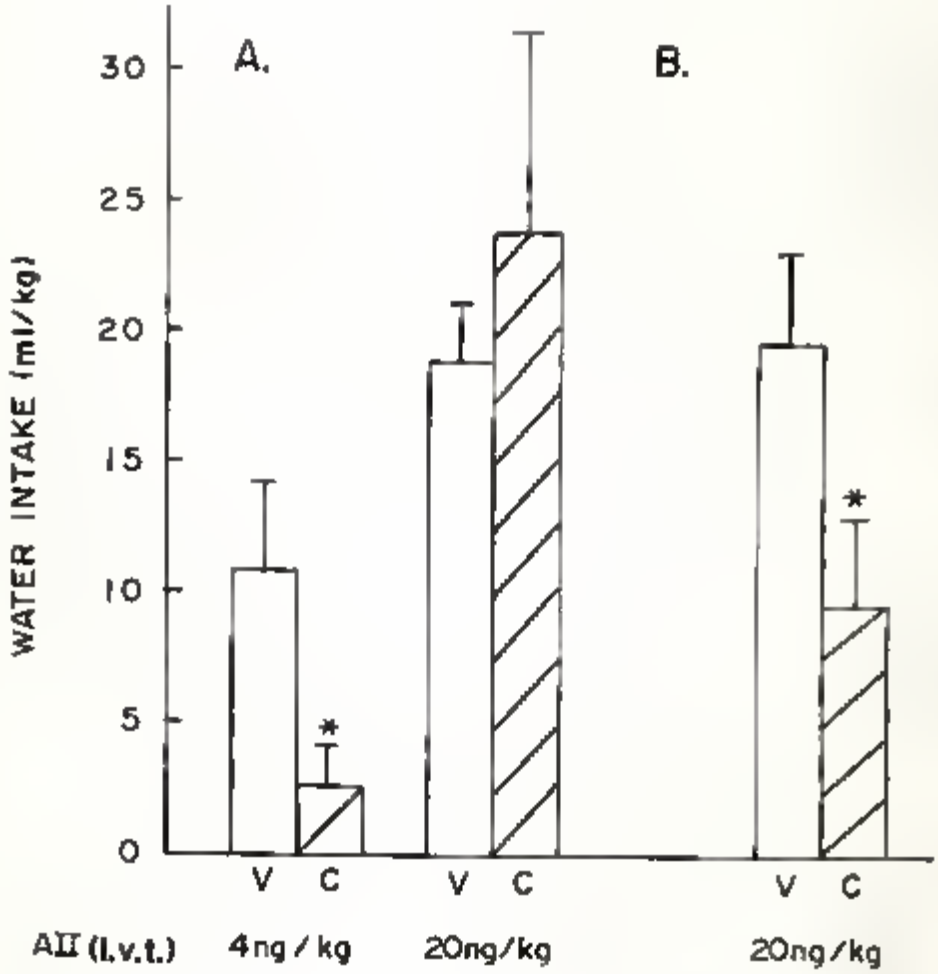
Methods

Female rats of the Blue Spruce Farms (Sprague-Dawley) strain weighing 180-280 g were used. On test days, the rats were weighed and injected with the appropriate drug or vehicle (details to be given with results). Each rat was placed into an individual stainless steel cage and water intakes were measured at 0.5, 1 and 2 hr after administration of the dipsogen. All experiments were set up according to a statistical factorial design to assess the effects of

Figure 3. Effect of graded doses of clonidine in combination with (A) AII (200ug/kg) and (B) isoproterenol (25 ug/kg) on water intake of rats during the first hr of administration (Modified from Fregly and Kelleher, 1980; Fregly, Kelleher and Greenleaf, 1981).

Water intake during first hour after administration of angiotensin II (ml / kg)
A.
Water intake during first hour after isoproterenol (ml/kg)
B.
Isoproterenol (25 µg/kg b.w., s.c.)
Dose of clonidine (µg/kg body wt.)

Figure 4. Effect of clonidine administered i.p. (12 ug/kg, panel A) and i.v.t. (8 ug/kg, panel B) and the corresponding vehicle (V) on water intake during the first 30 min after central administration of AII (either 4 or 20 ng/kg). One SE is set off at each mean. *Significantly different from control ($p<0.05$).


A.
B.
WATER INTAKE (ml/kg)
30
25
20
15
10
5
0
*
*
V C V C V C
AII (i.v.t.)
4ng / kg
20ng/kg
20ng/kg

treatment and interaction (Snedecor and Cochran, 1956). The doses of clonidine, naloxone and isoproterenol are expressed as their hydrochloride salt.

Results

Effect of Clonidine and Naloxone on Angiotensin II-induced Drinking

Twenty-four experimentally naive rats were randomly divided into four equal groups, each receiving three injections. Group 1 served as the control group and received two i.p. injections of the saline vehicle used to dissolve the naloxone and clonidine. Group 2 was administered clonidine (12 ug/kg, i.p.) and the saline vehicle; group 3 received naloxone (1 mg/kg, i.p.) and the saline vehicle; and group 4 received 12 ug clonidine/kg in combination with 1 mg naloxone/kg. Additionally, all four groups received AII (200 ug/kg, i.p.) ten minutes after the initial injections.

The administration of naloxone, clonidine and the combination of these two compounds significantly attenuated the AII-induced water intake ($p<0.01$) (Fig. 5). However, the effect of the drug combination was significantly greater than that of either agent alone ($p<0.05$). The interaction between the two treatments was not significant [$F(3,20) = 2.65$], suggesting additivity of their effects on drinking.

Effect of Clonidine and Naloxone on Isoproterenol-induced Drinking

This experiment was identical to the one above except that the dipsogen was D,L-isoproterenol (25 ug/kg, s.c.), and the dose of naloxone was 0.5 mg/kg. The isoproterenol-induced drinking was not significantly inhibited by these doses of either naloxone or clonidine. However, the combination of naloxone and clonidine produced a significant attenuation of drinking ($p<0.01$) (Fig. 6). The interaction between these two treatments was not significant [$F(3,20) = 1.45$], again suggesting additivity of their effects on drinking.

Figure 5. Effect of naloxone (1 mg/kg), clonidine (12 ug/kg) and clonidine + naloxone on AII-induced drinking (200 ug/kg). On SE is set offf at each mean. *Significantly different from saline-treated control group ($p<0.01$). **Significantly different from both saline-treated ($p<0.01$) and naloxone-treated ($p< 0.05$) groups.

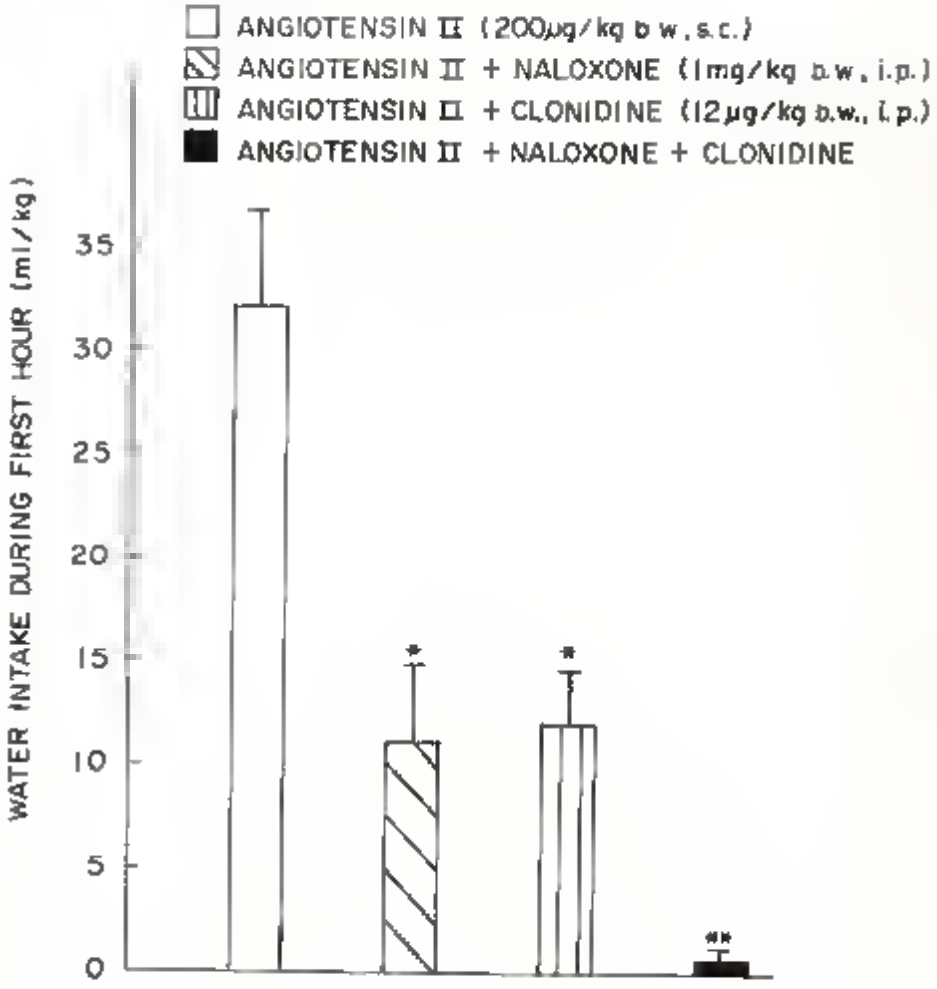
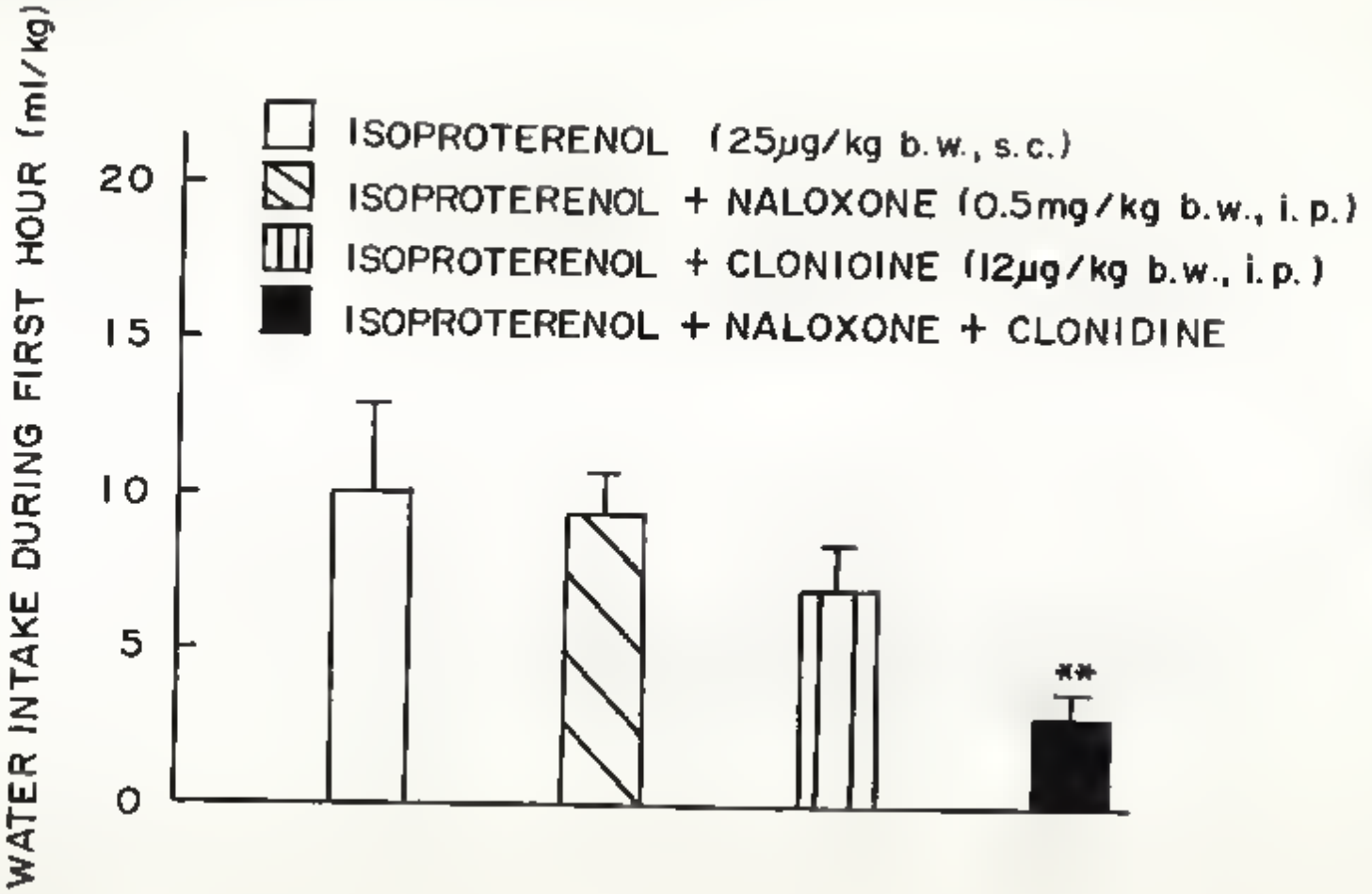

ANGIOTENSIN II (200µg/kg b.w., s.c.)
ANGIOTENSIN II + NALOXONE (1mg/kg b.w., i.p.)
ANGIOTENSIN II + CLONIDINE (12µg/kg b.w., i.p.)
ANGIOTENSIN II + NALOXONE + CLONIDINE
WATER INTAKE DURING FIRST HOUR (ml/kg)
35
30
25
20
15
10
5
0
*
*
**

Figure 6. Effect of naloxone (0.5 mg/kg), clonidine (6 ug/kg) and naloxone + clonidine on isoproterenol-induced drinking (25 ug/kg). *Significantly different from both saline-treated ($p<0.01$) and naloxone-treated ($p<0.05$) groups.


WATER INTAKE DURING FIRST HOUR (ml/kg)
20
15
10
5
0
ISOPROTERENOL (25μg/kg b.w., s.c.)
ISOPROTERENOL + NALOXONE (0.5mg/kg b.w., i.p.)
ISOPROTERENOL + CLONIDINE (12μg/kg b.w., i.p.)
ISOPROTERENOL + NALOXONE + CLONIDINE
**

Effect of Yohimbine and Naloxone on AII-induced Drinking

Three groups of six rats were used, each receiving three injections. Group 1 (control) received two injections of the saline vehicle. Group 2 was administered naloxone (1 mg/kg, i.p.) and saline, and group 3 received naloxone (1 mg/kg i.p.) and yohimbine (300 ug/kg, i.p.). Ten minutes later, all of the rats received AII (200 ug/kg, s.c.).

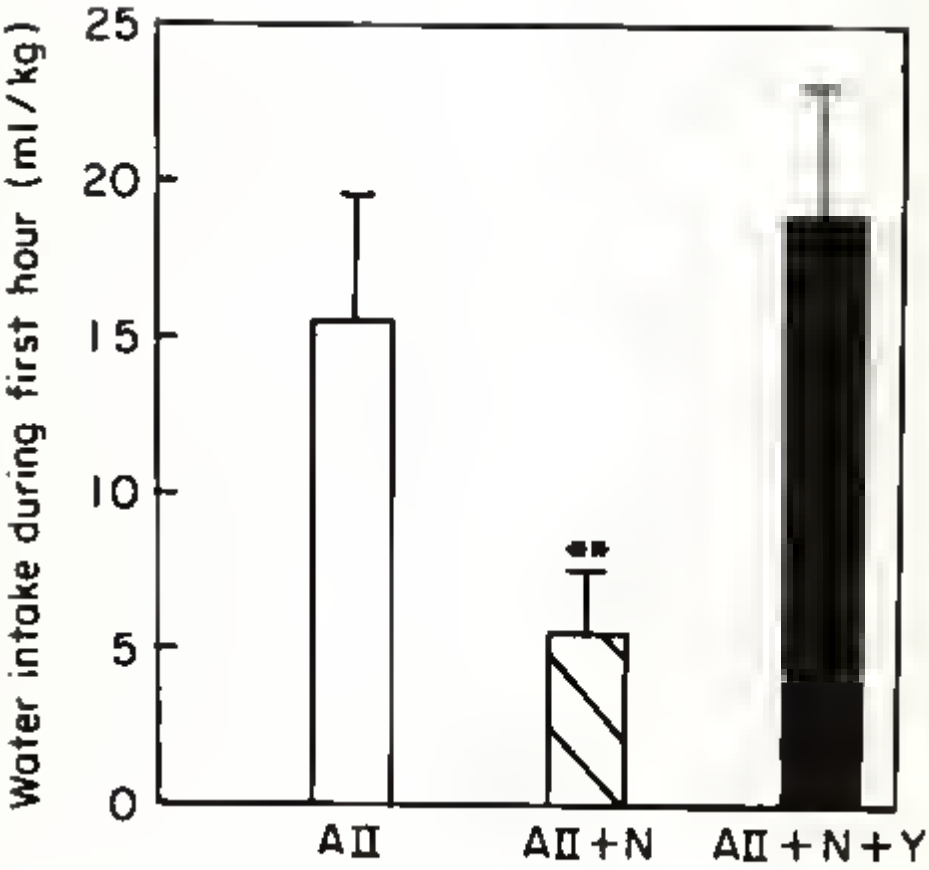
Naloxone again produced a significant attenuation of AII-stimulated drinking (Fig. 7), an effect which was reversed by the simultaneous administration of the alpha$_2$-adrenoceptor antagonist, yohimbine. Other experiments (Fregly et al., 1983) have shown that AII-induced drinking is enhanced by this dose of yohimbine.

Discussion

Naloxone and clonidine have been implicated in the attenuation of virtually all experimentally-induced drinking studied. The results of this study reveal that the combination of submaximal doses of the two antidipsogenic agents can suppress water intake induced by either AII or isoproterenol to a greater degree than either one separately. Since the sites of action of naloxone (to block opiate receptors) and clonidine (to inhibit the release of norepinephrine from the presynaptic alpha-adrenergic terminals) are presumed to be distinct and separate, an interactive attenuation of drinking would be expected. However, analysis of the inhibition by naloxone and clonidine on AII and isoproterenol-induced drinking revealed an additive suppression of water intake.

The participation of the alpha$_2$-adrenoceptor in drinking has been further substantiated by demonstrations that the specific alpha$_2$-agonist (clonidine) and alpha$_2$-antagonist (yohimbine) have opposite effects on

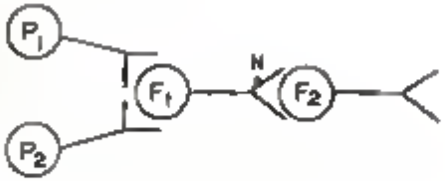
Figure 7. Effect of yohimbine (300 ug/kg) on the antidipsogenic effect of naloxone (1 mg/kg) on AII-induced drinking (200 ug/kg). **Significantly different from control ($p<0.01$).


Water intake during first hour (ml/kg)
25
20
15
10
5
0
**
AII
AII+N
AII+N+Y

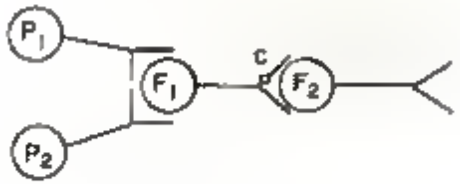
experimentally-induced drinking. To test the possible role of naloxone at the alpha-adrenergic receptor in the attenuation of AII-induced water intake, yohimbine, the alpha$_2$-adrenoceptor antagonist, was administered. When combined with naloxone, yohimbine reversed the antidipsogenic effect of naloxone on AII-induced drinking.

As the actions of naloxone have always been assumed to be opioid-specific, it was naturally inferred that opioids were involved in the mechanisms of drinking. Opioid agonists have been reported to increase water intake (Cooper, 1981; Sanger, 1981; Sanger and McCarthy, 1981). The experimental paradigm of Sanger and McCarthy, however, did not separate the drinking effects from the opiate-stimulated increase in food intake, so the actual effect of the opioids on unstimulated-drinking is unclear from their results. The effect of opioid agonists on experimentally-induced drinking is, however, more clearly defined. Enkephalins and endorphins attenuate both AII- and hypertonic saline-induced drinking through a naloxone-sensitive mechanism (Summy-Long et al., 1981a; Summy-Long et al., 1981b), and morphine can reduce drinking stimulated by carbachol (Chance and Rosencrans, 1977) and water-deprivation (Frenk and Rogers, 1979). It has been inferred from these results that the endogenous opioids are involved in both AII and osmoreceptor drinking pathways. It seems paradoxical that both opiate agonists and antagonists suppress experimentally-induced drinking. The models of Oatley (1973) and Toates (1979) have been built upon to provide a partial explanation for this paradox. The new model in Figure 8 involves a simplified diagram of two receptors (P_1 and P_2, which represent the two drinking pathways -- osmoreceptor and AII) and their convergence to form a final common pathway (F_1 and F_2). Since both naloxone and clonidine inhibit responses to stimulation of both drinking pathways (P_1 and P_2), then their actions are likely to be at

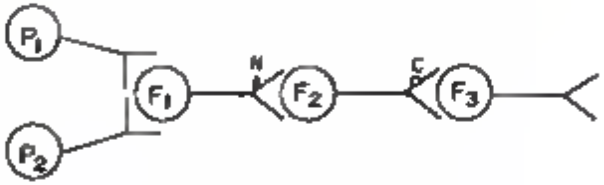
Figure 8. A model illustrating the possible sites of naloxone (N), clonidine (C), and endogenous opioids (O) along the final pathway involved in drinking responses. P_1 and P_2 represent the angiotensin and osmoreceptor pathways, respectively which converge into a final pathway (F_1, F_2 and F_3). NE=Norepinephrine.



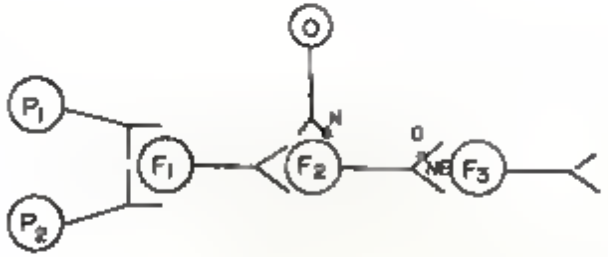
I.



II.



III.



IV.

some point on the final common pathway (I and II). Whether the sensitive sites are at the same terminal, or in series (as shown in III), cannot be determined at this time.

The fourth figure in the model (IV) considers the suppressive effects of the endogenous opioids (O) on drinking induced by stimulation of osmoreceptor and AII pathways. Analogous to the actions of naloxone and clonidine, enkephalins and beta endorphin may also exert their effect along the final common pathway. The possibility exists that alpha$_2$-adrenoceptors may be related to the opioid inhibition of experimentally-induced drinking. In addition, the antidipsogenic effect of naloxone in the absence of opioid influence may not be opiate-related but rather a consequence of alpha-adrenergic mediation. Thus, the inhibition of experimentally-induced drinking by naloxone, as well as the endogenous opioids, may be manifested by the inhibiton of norepinephrine (NE) release from the pre-synaptic alpha-adrenergic terminals. This suggestion must, however, remain speculative in the absence of any other evidence that the effects of either opioid peptides or naloxone involve direct mediation by alpha-adrenoceptors.

CHAPTER IV
ANGIOTENSIN II-INDUCED HYPOTHERMIA

Introduction

Body temperature is maintained by a balance between heat production and heat loss. A hypothalamic regulatory center in the brain mediates these responses and is influenced by the temperature of the blood entering it as well as by afferent nervous impulses from peripheral thermoreceptors (Jensen, 1980). The mediation of these responses by central "neurohumors" was first suggested by Von Euler (1961). The later studies of Feldberg and Myers (1963) and Myers and Yaksh (1969) suggested that the hypothalamic regulation of body temperature was mediated through a balance of neurohumors, particularly norepinephrine, acetylcholine, and serotonin. A number of peptides in the brain has also been implicated as modulators of thermoregulatory processes, either by acting directly in the hypothalamus or by influencing one of its neural pathways (Blatteis, 1982; Clark, 1979; Lipton and Glyn, 1980).

Angiotensin II, an octapeptide involved in the regulation of blood pressure, secretion of aldosterone from the adrenal cortex, and water balance (Reid et al. 1978), may also influence thermoregulation in mammals. Central administration of this compound was shown to reduce body temperature in monkeys, rabbits (Lin, 1980; Sharpe et al., 1979), and rats (Kiyohara et al., 1984). However, large doses were required to induce these responses, and the mechanisms underlying the hypothermic effects of centrally administered AII on body temperature were not clearly defined. AII exerts its effect on blood

pressure, secretion of aldosterone, and water balance by both peripheral and central mechanisms (Reid et al., 1978; Severs et al., 1971). While centrally administered AII can reduce body temperature, an effect of peripherally administered AII on body temperature has not been reported. This was the initial aim of these studies. When it was observed that peripherally administered AII induced hypothermia in rats, additional studies were carried out to identify some of the possible mechanisms mediating this effect.

The tail of the rat serves as a short-term thermoregulatory organ and in response to both internal and ambient temperature changes, the vasculature of the tail either vasoconstricts or vasodilates to maintain a constant body temperature (Hellstrom, 1975; Raman et al., 1983; Rand et al., 1965). Previous studies from this laboratory (Barney et al., 1979; Pregly, 1983) indicated that activation of beta-adrenoceptors by administration of isoproterenol is accompanied by an increase in tail skin temperature (TST) that is secondary to an increase in metabolic rate and heat production (Pregly et al., 1980). Since peripheral administration of AII also increased TST, its possible interaction in the response was studied by means of a beta-adrenergic agonist and antagonist. Further, the cholinergic system also mediates hypothermic responses (Hellon, 1972; Jensen, 1980; Myers and Yaksh, 1969); hence, the effect of cholinergic antagonists on the thermoregulatory responses following administration of AII was assessed.

Methods

Naive female rats of the Blue Spruce Farms (Sprague-Dawley) strain weighing 200-380g were used. The measurement of colonic (CT) and tail skin (TST) temperatures was performed as detailed in the general methods. Table I provides protocols for the seven different experiments described.

TABLE 1: DRUG TREATMENT PROTOCOL

EXP #	N	(GROUP #)	TREATMENT
1	40	(1)	AII (200 ug/kg, s.c.)
		(2)	AII (100 ug/kg, s.c.)
		(3)	AII (50 ug/kg, s.c.)
		(4)	AII (10 ug/kg, s.c.)
		(5)	0.9% Saline (1 ml/kg)
2	14	(1)	AII (200 ug/kg, s.c.)
		(2)	Saline (1 ml/kg)
3	24	(1)	Saline controls (1 ml/kg)
		(2)	Saline 15 min prior to AI (200 ug/kg, s.c.)
		(3)	Captopril (35 mg/kg, i.p.) 15 min prior to saline
		(4)	Captopril 15 min prior to AI
4	24	(1)	Saline controls (1 ml/kg)
		(2)	Saline + AII (200 ug/kg, s.c.)
		(3)	Isoproterenol (25 ug/kg, s.c.) + saline
		(4)	Isoproterenol + AII (simultaneously)
5	24	(1)	Saline controls (1 ml/kg)
		(2)	Saline 20 min prior to AI1 (200 ug/kg, s.c.)
		(3)	Propranolol (6 mg/kg, i.p.) 20 min prior to saline
		(4)	Propranolol 20 min prior to AII
6	24	(1)	Saline controls (1 ml/kg)
		(2)	Saline 15 min prior to AII (200 ug/kg, s.c.)
		(3)	Atropine sulfate (6 mg/kg, i.p.) 15 min prior to saline
		(4)	Atropine sulfate 15 min prior to AII
7	24	(1)	Saline controls (1 ml/kg)
		(2)	Saline 15 min prior to AII (200 ug/kg, s.c.)
		(3)	Atropine methyl nitrate (3.25 mg/kg, i.p.) 15 min prior to saline
		(4)	Atropine methyl nitrate 15 min prior to AII

Experiment 2 differed from the others and will be described separately. In this experiment, the rate of oxygen consumption was measured with an open circuit system and a Beckman OM-11 oxygen analyzer (Adolph et al., 1954). Each rat was placed into a cylindrical, water-jacketed, lucite chamber. The water temperature was adjusted to maintain the temperature inside the chamber at 29 $\pm$ 1°C. This higher temperature was chosen to mimic the response to restraint in air at 26°C. The higher temperature is also necessary to offset the greater conductive heat loss accompanying immersion experiments (Pregly et al., 1961). Room air passed through the chamber at a rate of 350 ml/min. The effluent air was dried and oxygen concentration measured. Oxygen consumption was calculated in terms of ml of oxygen consumed per min per kg body weight$^{0.75}$. Colonic and tail skin temperatures were measured with copper-constantan thermocouples as in the other experiments.

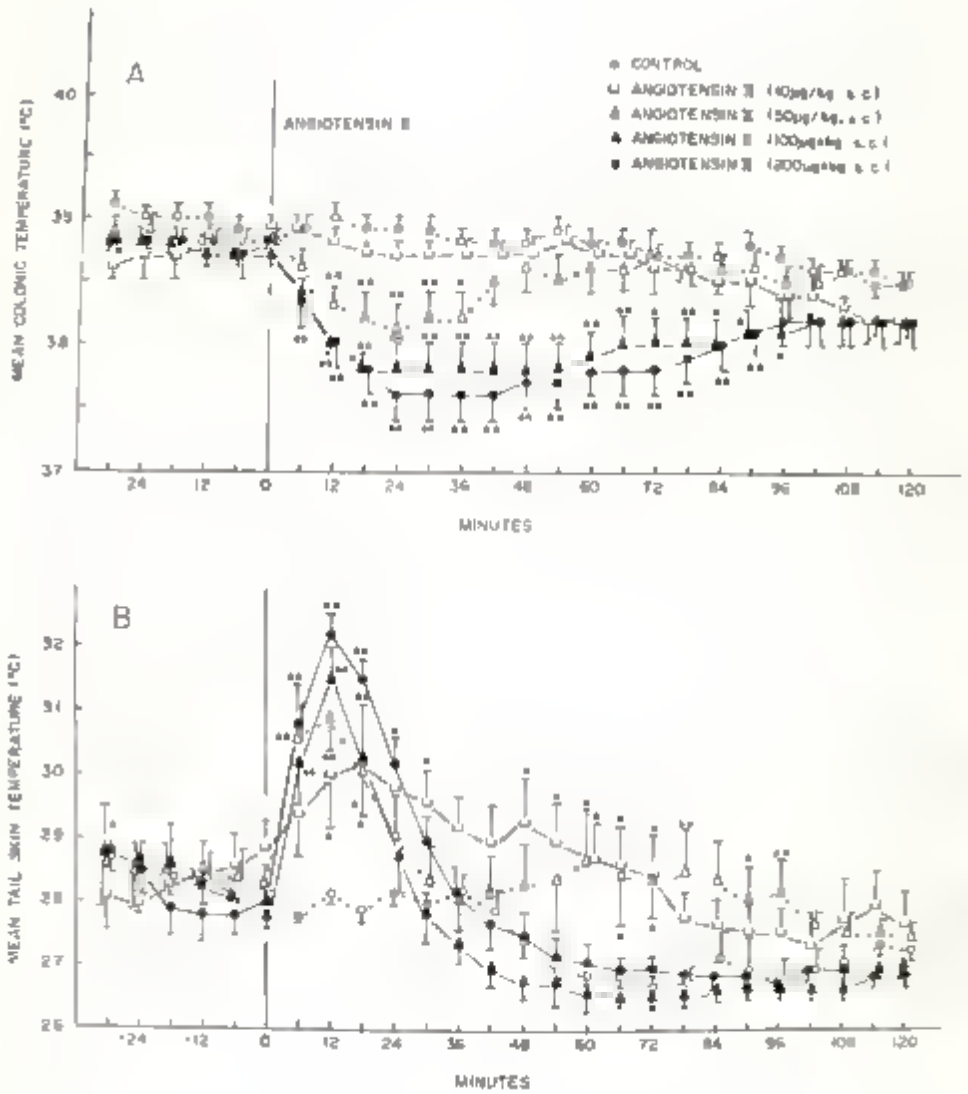
At the beginning of each experiment, the thermocouples were attached to the rats, and they were placed in the lucite chambers. Measurements of the temperature of the chamber, TST, CT, and oxygen consumption were made every 4 min during a 30 min control period. Seven of the rats were then administered 200 ug of AII/kg, s.c., while the remaining seven received an equal volume of the vehicle (1 ml/kg, s.c.). Immediately thereafter, the rats were placed back into the lucite chamber and measurements continued for another 96 min. Mean rates of oxygen consumption, TST and CT were calculated for each group and compared statistically by Student's t-test (Snedecor and Cochran, 1956).

Results

Experiment 1. Effect of Acute Administration of Angiotensin II on Tail Skin and Colonic Temperatures of Rats

Subcutaneous administration of AII induced a dose-dependent decrease in CT to a maximal level of -1.3°C (Fig. 9A). While the maximal fall in CT

Figure 9: Mean colonic (A) and tail skin (B) temperatures of rats administered either saline or AII (10, 50, 100, and 200 ug/kg, s.c.) at time zero are shown. One SE is set off at each mean.
*Significantly different from control ($p<0.05$).
**Significantly different from control ($p<0.01$).


A
ANGIOTENSIN II
CONTROL
ANGIOTENSIN II (40μg/kg s.c.)
ANGIOTENSIN II (50μg/kg s.c.)
ANGIOTENSIN II (100μg/kg s.c.)
ANGIOTENSIN II (200μg/kg s.c.)
MEAN COLONIC TEMPERATURE (°C)
40
39
38
37
24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
32
31
30
29
28
27
26
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES

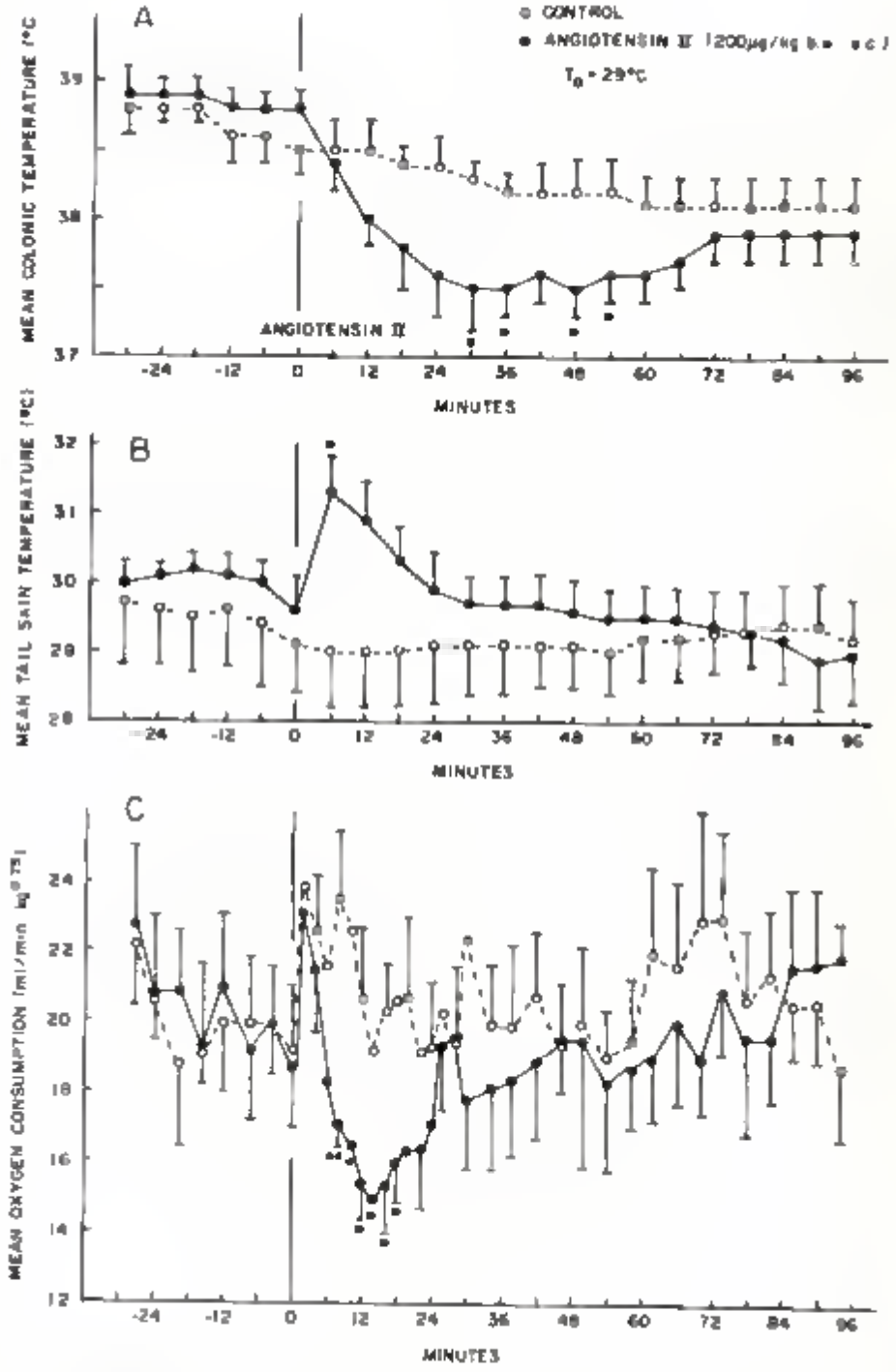
induced by 100 ug of AII/kg (-1.0°C) was less than that induced by the 200 ug dose (-1.3°), the difference was not significant. The duration of the response at each of these doses was also similar (84 min). However, the fall in CT accompanying administration of 50 ug of AII/kg (-0.6°) was significantly different from the higher doses as well as the lowest dose. The duration of the response was likewise reduced to 24 min. Administration of 10 ug AII/kg induced a decrease of 0.2° for the first 90 min which was not significantly different from controls. The response to the higher doses increased toward control level after 90 min, while the response accompanying 10 ug AII/kg began to decrease, so that by 120 min, the temperature had decreased 0.7°C.

The effect of AII on TST was similarly dose-dependent (Fig. 9B). The 100 and 200 ug/kg doses induced similar increases of 3.7° and 4.2°C, respectively. The maximal response occurred in 12 min and returned to control level by 24 min. TST responses to 10 and 50 ug/kg doses were significantly lower and of shorter duration than the higher doses. Increases of only 1.2 and 2.6°C, respectively, were observed. Although small, these responses were still significantly different from controls. The increase in TST accompanying administration of 10 and 50 ug of AII/kg remained above control levels for 96 min, suggesting a prolonged vasodilation and heat loss with the lower doses.

Experiment 2. Effect of Acute Administration of Angiotensin II on Rate of Oxygen Consumption in Rats

Administration of AII (200 ug/kg) significantly ($p<0.01$) decreased rate of oxygen consumption within 8 min for a duration of 16 min (Fig. 10C). The rate of oxygen consumption of the control group was somewhat variable, but a slight increase was noted within the first three min. This increase was probably due

Figure 10: Mean colonic temperature (A), tail skin temperature (B), and rate of oxygen consumption (C) of rats administered either saline or AII (200 ug/kg, s.c.) at time zero are shown. One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).


A
CONTROL
ANGIOTENSIN II (200µg/kg b.w. s.c.)
T_a = 29°C
MEAN COLONIC TEMPERATURE (°C)
39
38
37
ANGIOTENSIN II
-24
-12
0
12
24
36
48
60
72
84
96
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
32
31
30
29
28
MINUTES
C
MEAN OXYGEN CONSUMPTION (ml/min kg$^{0.75}$)
24
22
20
18
16
14
12
MINUTES

to the excitement accompanying the injection since the AII-treated group also showed a similar initial increase.

TST also significantly increased in the treated group, but not in the controls (Fig 10B). The less robust response of TST under these conditions can be attributed to the greater thermal stress of the rat within a chamber immersed in water compared with restraint in air (Fregly et al., 1961). The increase in TST was therefore only significant at 6 min. CT decreased 1.3°C in response to administration of AII (Fig. 10A). There was greater than usual variability in CT of the treated animals under these conditions and a slight progressive decrease in the CT of the control group.

Experiment 3. Effect of Administration of Captopril on the Responses of Tail Skin and Colonic Temperatures to Acute Administration of Angiotensin I (AI)

Administration of AI evoked changes in CT and TST that were similar to those following treatment with AII (Fig. 11). CT fell 1.0°C within 18 min after injection of AI and remained significantly reduced for 54 min. TST increased 5.9°C within 12 min and returned to control levels by 30 min. Administration of the AI converting enzyme inhibitor, captopril, had no effect on either CT or TST when compared to controls. Administration of captopril in combination with AI, on the other hand, completely abolished both the CT and TST responses induced by treatment with AI.

Experiment 4. Effect of Simultaneous Administration of Angiotensin II and Isoproterenol on Tail Skin and Colonic Temperatures

Isoproterenol and AII were administered together in order to evaluate their combined effects on thermoregulatory responses. The combination resulted in a mean CT which was not significantly different from controls (Fig. 12A). This suggests thet isoproterenol prevented the AII-induced fall in CT.

Figure 11: Effect of captopril (35 mg/kg, i.p.) on mean colonic (A) and tail skin (B) temperatures following administration of AI (200 ug/kg, s.c.). Captopril was administered 15 min prior to administration of AI. One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

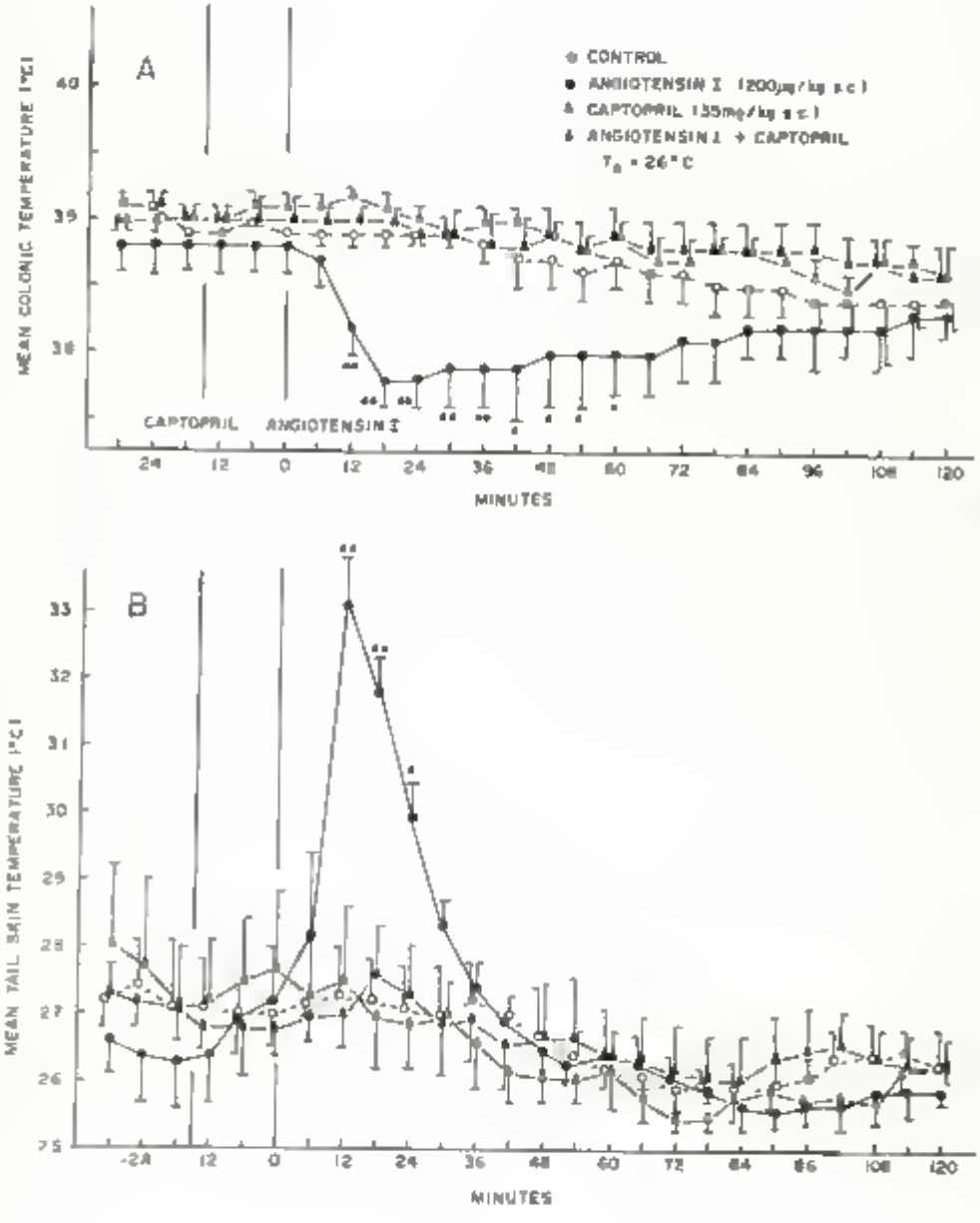
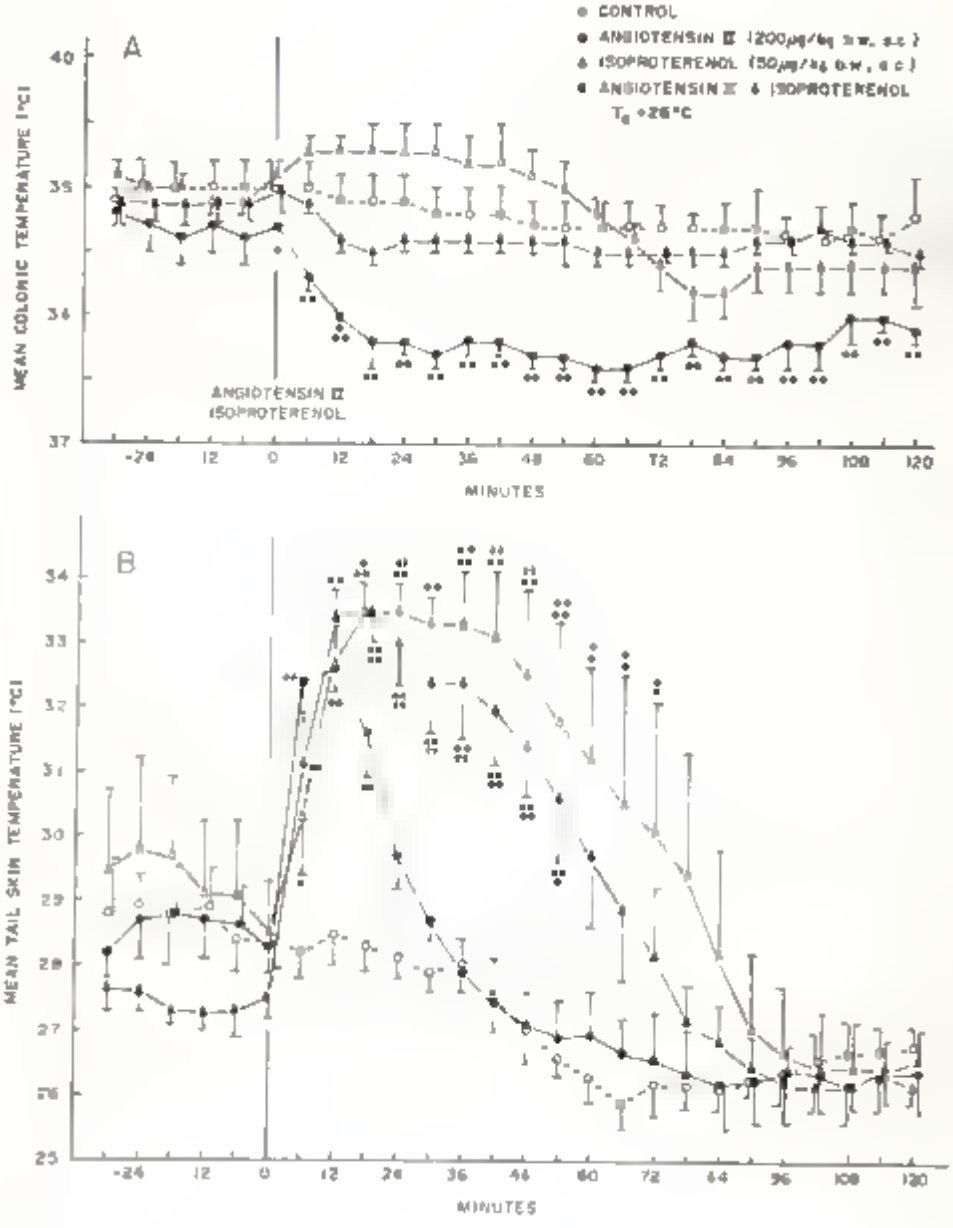

A
CONTROL
ANGIOTENSIN I (200µg/kg s.c.)
CAPTOPRIL (35mg/kg s.c.)
ANGIOTENSIN I + CAPTOPRIL
T_a = 26°C
MEAN COLONIC TEMPERATURE (°C)
40
39
38
CAPTOPRIL
ANGIOTENSIN I
-24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
33
32
31
30
29
28
27
26
25
-24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES

Figure 12. Mean colonic (A) and tail skin (B) temperatures following administration of isoproterenol (50 ug/kg, s.c.), AII (200 ug/kg, s.c.), and the combined administration of AII and isoproterenol at time zero. One SE is set off at each mean. * Significantly different from control ($p<0.05$). ** Significantly different from control ($p<0.01$). ‡Isoproterenol + AII significantly different from AII ($p<0.05$). ‡‡Isoproterenol + AII significantly different from AII ($p<0.01$).


CONTROL
ANGIOTENSIN II (200μg/kg b.w., s.c.)
ISOPROTERENOL (50μg/kg b.w., s.c.)
ANGIOTENSIN II & ISOPROTERENOL
T_a +26°C
A
MEAN COLONIC TEMPERATURE (°C)
40
39
38
37
ANGIOTENSIN II
ISOPROTERENOL
-24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
34
33
32
31
30
29
28
27
26
25
-24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES

Treatment with isoproterenol alone caused a slight but insignificant rise in CT within 6 min, followed by a decrease in temperature below the level of controls.

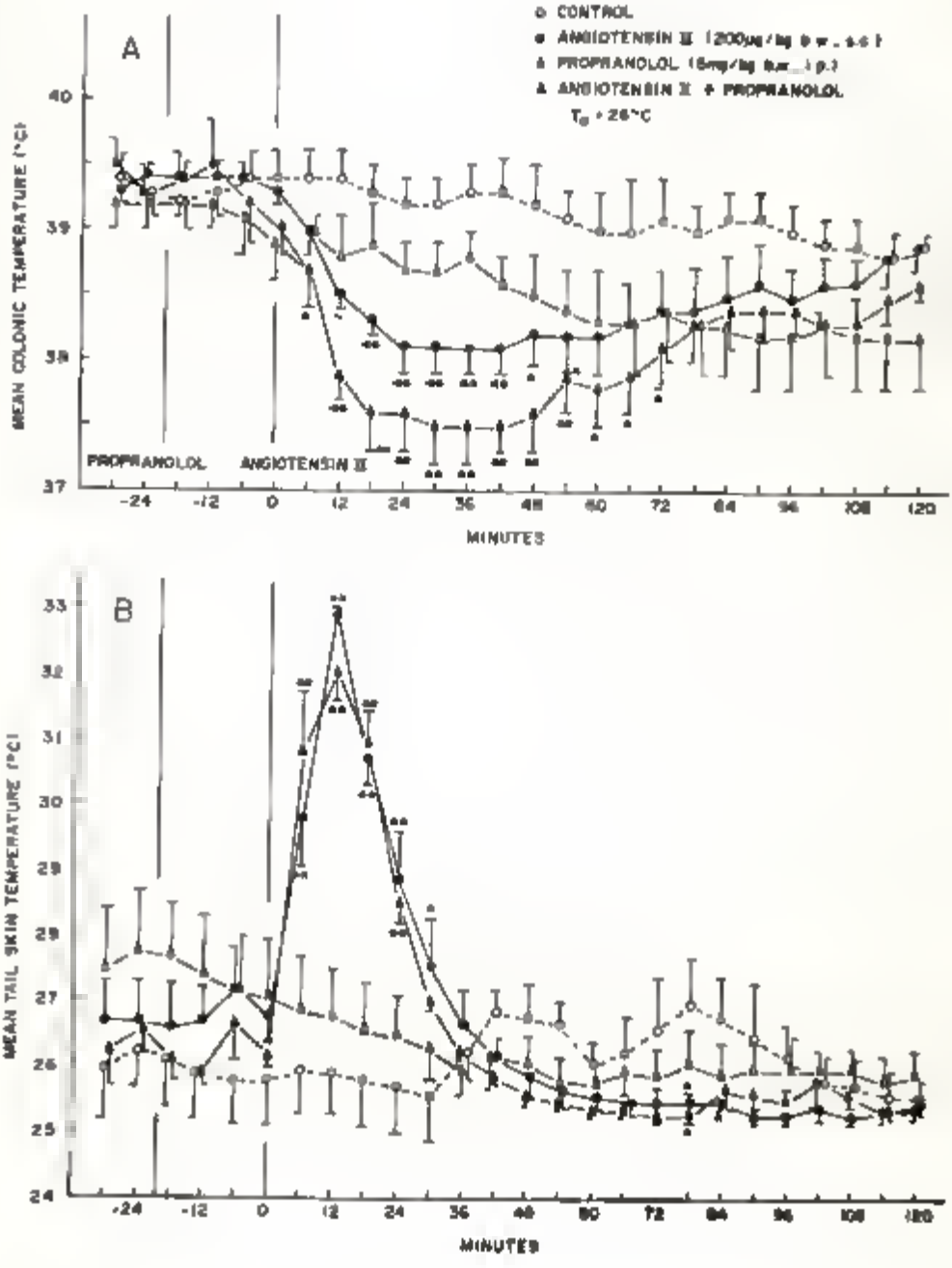
With respect to TST, administration of isoproterenol to saline-treated rats induced a significant elevation (5.7°C) for 70 min (Fig. 12B). The combination of AII and isoproterenol resulted in a maximal increase in TST which was similar to administration of isoproterenol alone. The duration of response accompanying the combined treatment was significantly reduced at 52 min. The maximal response to administration of AII (4.3°) occurred in 12 min while the maximal response to isoproterenol alone and isoproterenol + AII occurred at 18 min. After 18 min, TST's of the latter two groups were significantly different from the group treated with AII alone.

Experiment 5. Effect of Pretreatment with Propranolol on the Responses of Tail Skin and Colonic Temperatures to Acute Administration of Angiotensin II

Administration of the beta-adrenoceptor antagonist, propranolol, alone evoked a slow steady decrease in CT, and when administered prior to AII, propranolol enhanced the AII-induced fall in CT. Due to the variability between groups, this reduction was not significantly different from that due to treatment with AII alone (Fig. 13A). However, if the change in CT of the propranolol + AII-treated group was calculated from the time propranolol was administered, the maximal fall in temperature was 2.2°C which was significantly different from the mean maximal reduction of 1.2°C in the AII-treated rats.

The response of TST to administration of AII was not affected by pretreatment with propranolol (Fig. 13B).

Figure 13. Effect of propranolol on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Propranolol (6 mg/kg, i.p.) was administered 20 min prior to administration of angiotensin II (200 ug/kg, s.c.). One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).


A
CONTROL
ANGIOTENSIN II (200ng/kg b.w., s.c.)
PROPRANOLOL (6mg/kg b.w., i.p.)
ANGIOTENSIN II + PROPRANOLOL
T_a = 26°C
MEAN COLONIC TEMPERATURE (°C)
PROPRANOLOL
ANGIOTENSIN II
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
MINUTES

Experiment 6. Effect of Administration of Atropine Sulfate on the Responses of Tail Skin Temperature to Acute Administration of Angiotensin II

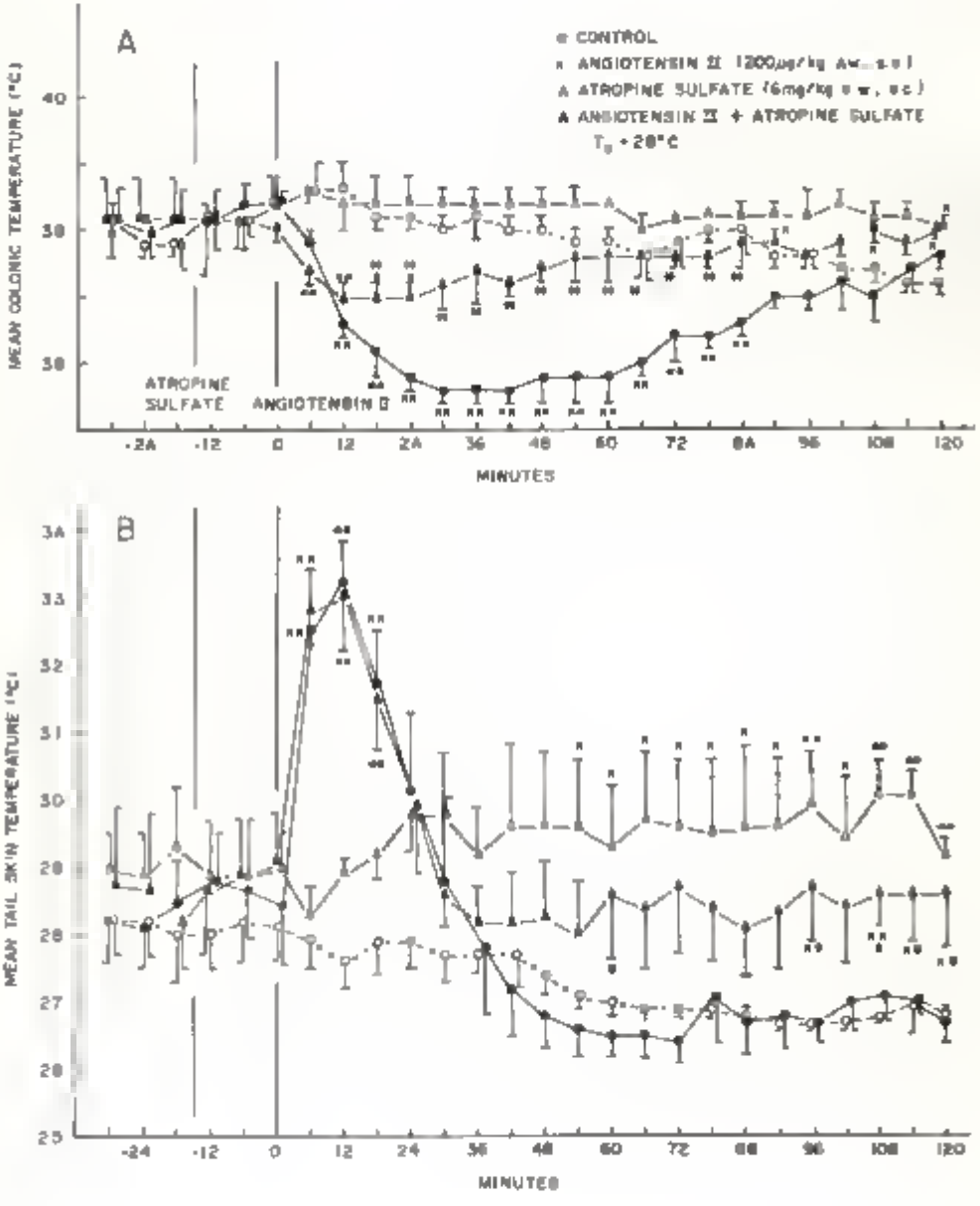
Pretreatment of rats with the cholinergic antagonist, atropine sulfate, significantly attenuated the fall in CT accompanying administration of AII (Fig. 14A). The combined treatment resulted in a 0.5°C reduction in temperature, which was significantly different from the control group for 20 min. Administration of atropine sulfate to saline-treated rats increased CT slightly (0.4° - 0.5°C), but since a slight increase was also seen in the control group, the difference between the groups was not significant. The CT of the control group slowly decreased over the 2 hr period, while the CT of the atropine-treated group remained elevated. By 2 hr, the two groups were significantly different.

The mean TST of the atropine-treated rats significantly increased over the 2 hr period (Fig. 14B). Administration of atropine sulfate prior to AII did not affect the increase in TST characteristic of administration of AII; however, treatment with atropine did interfere with the return of TST to control level. In the last 24 min of the experiment, the TST of rats treated with atropine + AII was significantly elevated above that of control and AII-treated rats.

Experiment 7. Effect of Administration of Atropine Methyl Nitrate on the Responses of Tail Skin and Colonic Temperature to Acute Administration of Angiotensin II

To evaluate central participation of the cholinergic system in temperature responses evoked by AII, 3.25 mg of atropine methyl nitrate/kg, which does not cross the blood-brain barrier, was administered prior to AII. Pretreatment with atropine methyl nitrate had no effect on either the decrease in CT or the increase in TST following administration of AII, but did interfere with the

Figure 14. Effect of atropine sulfate on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Atropine sulfate (6 mg/kg, i.p.) was administered 15 min prior to administration of AII (200 ug/kg, s.c.). One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$). ŦAtropine + AII significantly different from AII ($p<0.05$). ŦŦAtropine + AII significantly different from AII ($p<0.01$)


A
CONTROL
ANGIOTENSIN II (200μg/kg i.v., s.c.)
ATROPINE SULFATE (6mg/kg i.v., s.c.)
ANGIOTENSIN II + ATROPINE SULFATE
T_a = 28°C
MEAN COLONIC TEMPERATURE (°C)
ATROPINE SULFATE
ANGIOTENSIN II
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
MINUTES

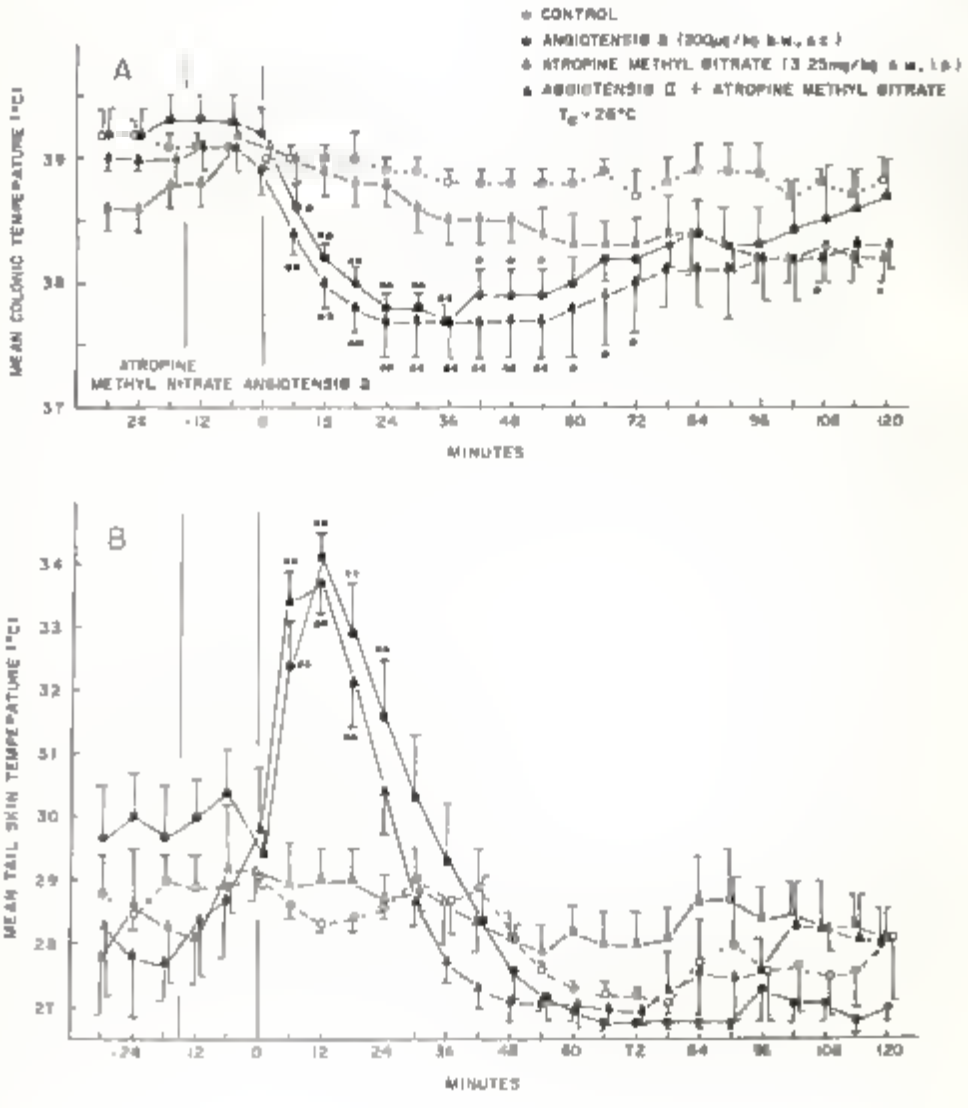
return of CT back to control levels (Fig. 15). Administration of atropine methyl nitrate to saline-treated rats induced a slow fall in CT which was significantly below that of controls by 2 hr but did not affect TST.

Discussion

Systemic administration of AII to rats evoked a significant fall (1.3°C) in CT, a significant increase (4.2°C) in TST, and a concomitant decrease in metabolic rate. The hypothermic response lasted up to 100 min, was specific for AII, and was dose-related. The hypothermic response to AI was inhibited when its conversion to AII was prevented by captopril (Fig. 11). Further, the extent of the hypothermic response was directly related to the dose of AII administered (Fig. 9). Thus, peripheral administration of AII evinced physiological responses in rats that characteristically occur when the animal is threatened with an elevation of body temperature. The reduction in heat production and activation of heat loss mechanisms suggest hypothalamic mediation. (Jensen, 1980; Maickel, 1970; Meeter, 1971; Myers and Yaksh, 1969).

An effect of central administration of AII on body temperature has been reported (Kiyohara et al., 1984; Lin, 1980; Lin et al., 1980; Sharpe et al., 1979). In the rabbit, hypothalamic microinjections of doses up to 1.5 ug of AII resulted in only a 0.5°C reduction in CT which occurred 15-30 min after administration and slowly returned to control levels within 75 min (Sharpe et al., 1979). Lin reported a 1.0-1.5°C decrease in the CT of rabbits (Lin, 1980) and rats (Lin et al., 1980) following intraventricular (i.v.t.) administration of AII, but his doses of 10-40 ug probably initiated a nonspecific effect and may actually have been toxic when one considers that only nanogram doses are required to induce significant increases in blood pressure and copious drinking in the rat. The doses used by Lin et al. (1980) also resulted in vasoconstriction. However, a small vasodilatory response is

Figure 15. Effect of atropine methyl nitrate on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Atropine methyl nitrate (3.25 mg/kg, i.p.) was administered 15 min prior to administration of AII (200 ug/kg, s.c.). One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$)


CONTROL
ANGIOTENSIN II (300µg/kg b.w., s.c.)
ATROPINE METHYL NITRATE (3.25mg/kg b.w., i.p.)
ANGIOTENSIN II + ATROPINE METHYL NITRATE
T_a = 26°C
A
MEAN COLONIC TEMPERATURE (°C)
ATROPINE METHYL NITRATE
ANGIOTENSIN II
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
MINUTES

also apparent in their data, although no mention is made of it. Thus, there appear to be differences between the responses to central and peripheral administration of AII that will require further investigation.

An additional study with rats has been reported in which Kiyohara et al. (1984) iontophoretically stimulated 17 out of 37 warm unit neurons in the medial preoptic area of the hypothalamus of the rat with AII and proposed that the increased activity of the warm units could explain the 1.0°C fall in CT induced by bilateral injection of AII into the medial prepoptic area (0.8 ug). However, the time-course of the hypothermic response in the study by Kiyohara et al. contrasts with the durations observed in the study of Lin et al. and in this study. In addition, the hypothermic response is not specific for AII since it accompanies hypothelamic administration of other peptides (eg., ACTH, MSH, substance P, neurotensin and bombesin) and lasts from 1-2 hr (Blatteis, 1982; Clark, 1979). Thus, whether there is a direct central effect of AII on temperature in the anterior hypothalamus is not clear at present. To explore alternative possibilities, the interaction of AII with the adrenergic and cholinergic systems was studied.

Administration of isoproterenol, a beta-adrenoceptor agonist, induces an increase in TST that may be secondary to an increase in metabolic rate and heat production (Fregly et al., 1980). In the present study, isoproterenol reversed the decrease in CT induced by administration of AII. However, the combined treatment maintained the elevation in TST for a longer period than was the case when AII was administered alone, and the vasodilatory effect of isoproterenol appeared to override the response to administration of AII. The influence of Isoproterenol on the CT responses could suggest the involvement of the beta-adrenergic system in the AII-induced hypothermia and may be related to the opposing actions of AII and isoproterenol on metabolic rate.

Previous investigations have also demonstrated that propranolol, a beta-adrenoceptor antagonist, could completely block the responses of CT and TST accompanying administration of isoproterenol (Fregly et al., 1980). Administration of propranolol prior to AII enhanced the fall in CT, but exerted no influence on TST. Since norepinephrine is proposed to stimulate mechanisms for heat production and conservation (Hellon, 1972; Jensen, 1980; Maickel, 1970), a tendency for propranolol to augment the fall in CT in the AII-treated rats might have been expected. However, the absence of an effect on TST presents two interpretations as to the intervention of the beta-adrenergic system in AII-induced hypothermia: (a) the inability of propranolol to affect the hypothermic response to administration of AII suggests that this response is not mediated through the beta-adrenergic system and that the interaction of AII and isoproterenol on CT responses is due entirely to their opposing effects on metabolic rate, and (b) the mechanisms of heat production and heat loss associated with the AII-induced hypothermia are mediated through separate neural pathways. The decrease in CT in response to treatment with AII appears to be mediated through the beta-adrenergic pathways since isoproterenol inhubited, and propranolol enhanced the fall in CT accompanying administration of AII. Present data do not allow us to choose one of these possibilities over the other.

In contrast to the role of norepinephrine in thermoregulation, cholinergic stimulation is proposed to mediate hypothalamic responses that initiate hypothermia in the rat (Hellon, 1972; Jensen, 1980; Myers and Yaksh, 1969). Central administration of carbachol induces a fall in CT which is manifested by decreased heat production and vasodilation (Meeter, 1971). Due to the similarity of the hypothermic responses induced by AII and carbachol, and the evidence that AII can stimulate the release of acetylcholine from pre- and

postganglionic neurons (Paulsset, 1967), an attempt was made to determine the extent of cholinergic mediation of the AII-induced hypothermia. The muscarinic receptor antagonist, atropine sulfate, significantly inhibited the reduction in CT accompanying administration of AII, but did not affect the increased TST. Atropine sulfate has been shown to reverse both the CT and TST responses to central carbachol administration (Kirkpatrick and Lomax, 1967). Atropine methyl nitrate, the quaternary analogue of atropine sulfate which does not cross the blood-brain barrier, was ineffective in altering any of the hypothermic responses to AII, which implies that the reduction in CT following treatment with AII may be a centrally mediated cholinergic response.

The vasodilatory response of the tail of the rat to administration of AII cannot be explained at this time. The possibility exists that the response is mediated either peripherally or centrally by a direct effect on AII receptors. It is of interest that hypothalamic microinjections of either acetylcholine + physostigmine or carbachol in the rabbit induces either hyperthermia (Sharpe et al., 1979) or no thermal response (Hellon, 1972). Referring back to the study of Sharpe et al. (1979) in which AII induced a 0.5^{o}C reduction in temperature, it may be speculated that this decrease is due to the heat loss component of the system, and that the cholinergic pathway may mediate the decrease in heat production observed in the present study. It is therefore possible that stimulation of central AII receptors may mediate heat loss mechanisms.

The separation of the hypothalamic regulatory mechanisms presents a puzzling aspect of the AII-induced hypothermia. It has been postulated that the mechanisms controlling heat loss and heat production are linked through the same hypothalamic control system. The concomitant but opposite responses of decreased heat production and increased heat loss to administration of AII are

responsible for the reduction in CT. The fact that a cholinergic blocking agent failed to affect the vasodilatory response of the tail to administration of AII, while preventing the reduction in colonic temperature, suggests that the component of the AII response affecting heat production can be blocked independently of that subserving heat loss. Additional studies will be required to clarify completely the mechanism by which peripheral administration of AII induces hypothermia in rats.

CHAPTER V
FACTORS AFFECTING ANGIOTENSIN II-INDUCED HYPOTHERMIA

Introduction

The role which angiotensin II (AII) plays in the regulation of blood pressure and water balance and the pathways mediating these responses have been thoroughly investigated. However, the mechanism by which AII induces its hypothermic effects is unknown. In order to identify further the mechanism of AII-induced hypothermia, several experiments were undertaken to evaluate this response in comparison to the AII-induced dipsogenic and pressor responses.

The effect of AII on blood pressure and drinking is thought to be mediated in part by catecholamines. Blockade of norepinephrine release with clonidine, an $alpha_2$-adrenoceptor agonist, can attenuate the dipsogenic response to AII (Fregly et al., 1981; Fregly et al., 1984a,b). Naloxone, an opioid receptor antagonist, also reduces the drinking response to AII (Brown and Holtzman, 1981b). As demonstrated in Chapter I, yohimbine, an $alpha_2$-adrenoceptor antagonist, can reverse the dipsogenic inhibition induced by both clonidine and naloxone, as well as potentiate the effect of AII on water intake (Fregly et al., 1984a,). Additionally, clonidine can reverse the pressor response of AII (Reid et al., 1978). Thus, the effect of these agents on AII-induced hypothermia was also evaluated.

Although a few studies have demonstrated that central administration of AII can affect temperature, large doses were used and the responses were not consistent (Lin et al., 1980; Sharpe et al., 1979). Therefore, the effects of I.V.T. administration of AII in doses comparable to those which induce dipsogenic and pressor responses were evaluated. In addition, to determine the

specificity of the response and to assure that the cause of the hypothermic response was not due to conversion of AII to AIII, the effects of specific angiotensin antagonist, saralasin, and of the heptapeptide were also investigated. In a final attempt to characterize the vasodilation of the tail induced by AII, indomethacin was administered to block the possible induction of AII-stimulated prostaglandin synthesis and release (Gumbrone and Alexander, 1975). The results of these studies are presented below.

Methods

Naive female rats of the Blue Spruce Farms (Sprague-Dawley) strain weighing 200-280 g were used.

Tail skin (TST) and colonic temperatures (CT) were measured at 26 ± 1°C while the rats were restrained in plexiglass tunnel-type cages as described in the general methods. Table 2 provides the protocols for the experiments described. For brevity, several of the experiments were grouped together in the table and in graphs.

Results

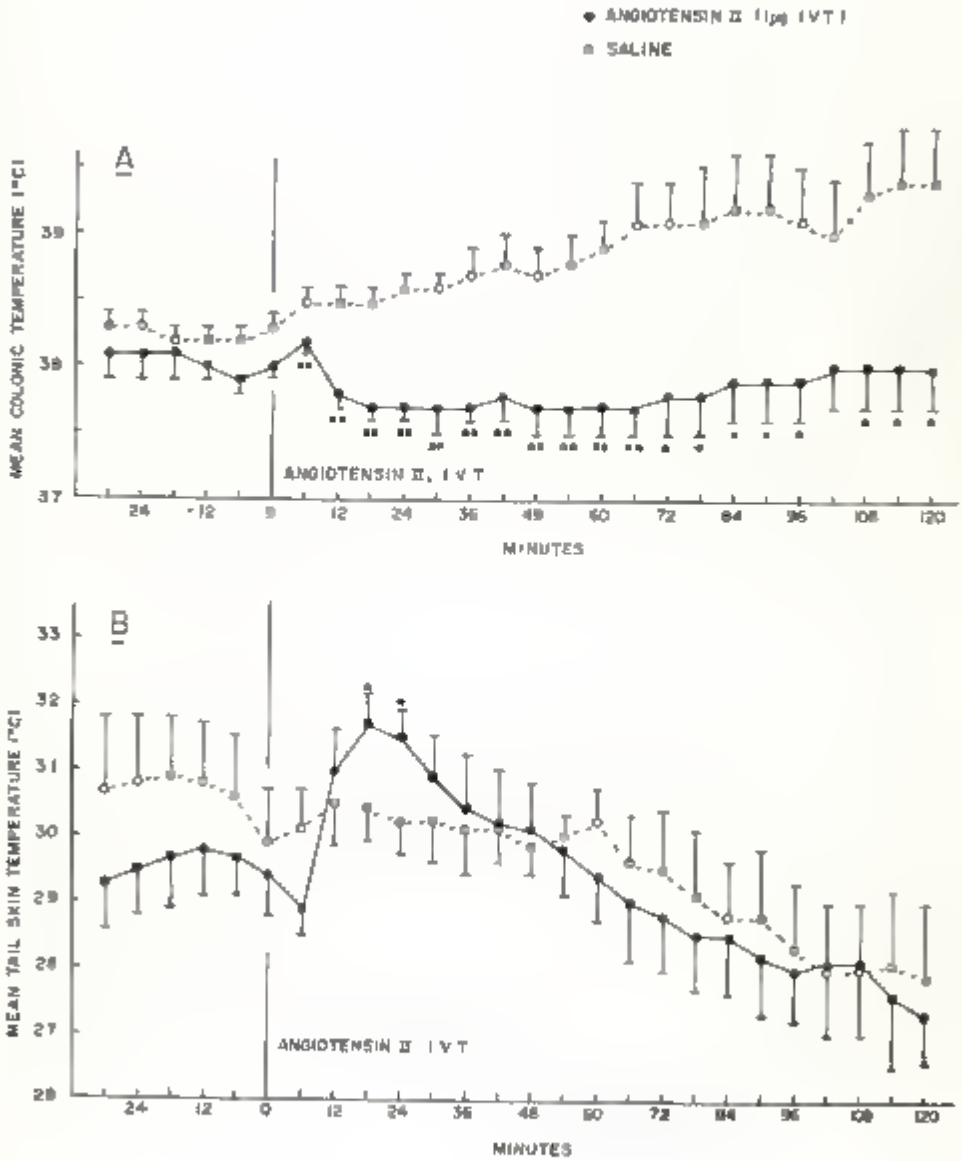
Experiment 1. Effect of Intracerebroventricular Administration of Angiotensin II on Tail Skin and Colonic Temperatures of Rats

Intracerebroventricular administration of 10 and 100 ng of AII did not significantly affect either CT or TST, while 1 ug of AII induced a maximal fall in CT of 0.4°C which was significantly different from controls for the duration of the experiment. CT of the control rats receiving saline (i.v.t.) steadily increased by 0.9°C at 120 min (Fig. 16A). The initial response of TST to i.v.t. administration of 1 ug of AII was a fall (0.5°C) in the first 6 min, followed at 18 min by an increase (2.4°C) in TST (Fig. 16B). This latter response was quantitatively different from that induced by s.c.

TABLE 2: DRUG TREATMENT PROTOCOL

EXP #	N	(GROUP #)	TREATMENT
I	24	(1)	AII (10 ng/5ul, i.v.t.)
		(2)	AII (100 ng/5ul, i.v.t)
		(3)	AII (1 ug/5ul, i.v.t.)
		(4)	Saline (5 ul, i.v.t.)
2	I2	(1)	AIII (200 ug/kg, s.c.)
		(2)	Saline (1 ml/kg)
3	66	(1)	Saline controls (1 ml/kg)
		(2)	Saline 10 min prior to AII (200 ug/kg, s.c.)
		(3-6)	Saralasin (either 1, 10, or 100 ug/kg, s.c.) 10 min prior to saline
		(7-9)	Saralasin (either 1, 10, or 100 ug/kg, s.c) 10 min prior to AII (200 ug/kg)
		(10)	Saline 10 min prior to AII (100 ug/kg, s.c.)
		(11)	Saralasin (100 ug/kg) 10 min prior to AII (100 ug/kg)
4A	36	(1)	Saline controls (1 ml/kg)
		(2)	Saline + AII (200 ug/kg, s.c.)
		(3-4)	Either clonidine (25 ug/kg, s.c.) or naloxone (1 mg/kg, i.p.) + saline
		(5-6)	Either clonidine or naloxone + AII (simultaneously)
4B	24	(1)	Saline controls (I ml/kg)
		(2)	Saline 15 min prior to AII (200 ug/kg, s.c.)
		(3)	Yohimbine (300 ug/kg, s.c.) I5 min prior to saline
		(4)	Yohimbine I5 min prior to AI1
4C	36	(1)	Saline controls (1 ml/kg)
		(2)	Saline 20 min prior to AI1 (200 ug/kg, s.c.)
		(3)	Indomethacin (4 mg/kg, i.p.) 20 min prior to saline
		(4)	Indomethacin (6 mg/kg, i.p) 20 min prior to saline
		(5)	Indomethacin (4 mg/kg) 20 min prior to AII
		(6)	Indomethacin (6 mg/kg) 20 min prior to AII

Figure 16. Mean colonic (A) and tail skin (B) temperatures of rats administered either i.v.t. saline or AII (1 ug, i.v.t) at time zero are shown. One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).


● ANGIOTENSIN II (1μg I V T)
○ SALINE
A
MEAN COLONIC TEMPERATURE (°C)
39
38
37
ANGIOTENSIN II, I V T
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
33
32
31
30
29
28
27
26
ANGIOTENSIN II I V T
24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES

administration of AII which resulted in a rapid (4-5°C) increase in TST within 6-12 min.

Experiment 2. Effect of Acute Administration of Angiotensin III on Tail Skin and Colonic Temperatures of Rats

Subcutaneous administration of AIII (200 ug/kg) induced a slight increase in CT (0.3°C) which was not significantly different from controls (Fig. 17A). However, as the CT of controls slowly decreased, CT of the AIII-treated group remained steady so that by 120 min the difference between the two groups was significant. The response of TST to administration of AIII was not significantly different from the controls, although an initial tendency for a decrease in TST was observed (Fig. 17B).

Experiment 3. Effect of Pretreatment with Saralasin on the Responses of Tail Skin and Colonic Temperatures to Acute Administration of Angiotensin II

Subcutaneous administration of 200 ug AII/kg produced a 1.2°C fall in CT and a 4.3°C increase in TST as previously reported (Figs. 18 and 19). The administration of 1 to 100 ug saralasin/kg did not significantly affect colonic temperature (Fig. 18A and 19A). In combination with AII, neither the 1 nor the 10 ug/kg dose of saralasin prevented the fall in CT induced by administration of AII, although CT of the group given 1 ug saralasin + AII returned to control levels at a faster rate than that of the group treated with AII alone. Pretreatment with 100 ug saralasin/kg, on the other hand, significantly attenuated the maximal fall in CT and the duration of the hypothermia induced by AII (Fig. 19A). The combined treatment resulted in a fall of only 0.7°C.

Although an agonist-like increase in TST of 1.2 and 1.9°C was induced by 1 and 10 ug saralasin/kg, respectively, the elevation in TST accompanying

Figure 17. Mean colonic (A) and tail skin (B) temperatures of rats administered either saline or AIII (200 ug/kg, s.c.) at time zero are shown. One SE is set off at each mean. *Significantly different from control ($p<0.05$).

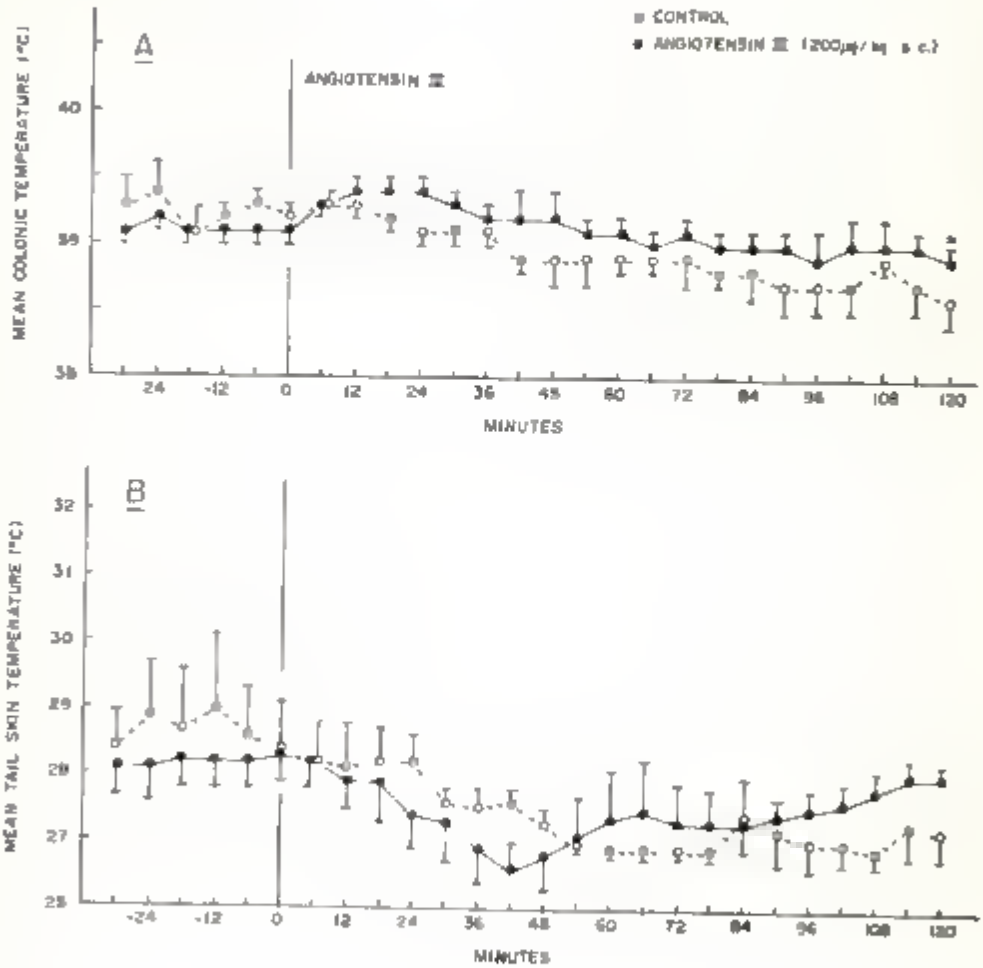

A
CONTROL
ANGIOTENSIN II (200μg/kg s.c.)
ANGIOTENSIN II
MEAN COLONIC TEMPERATURE (°C)
40
39
38
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
32
31
30
29
28
27
26
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES

Figure 18. Effect of 1 and 10 ug saralasin/kg, s.c. on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Saralasin was administered 10 min prior to AII (200 ug/kg, s.c.). One SE is set off at each mean.
†Significantly different from AII ($p<0.05$).
‡Significantly different from AII ($p<0.01$).

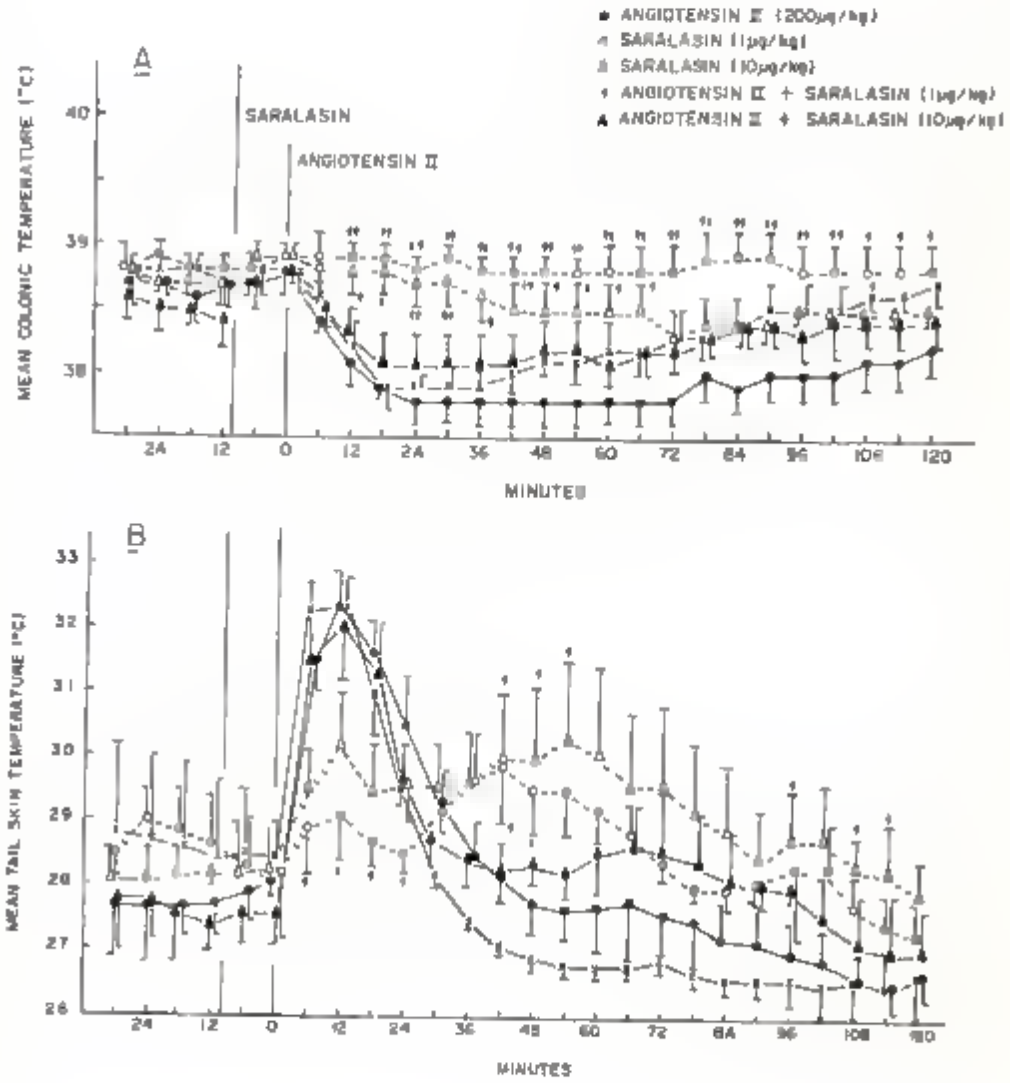
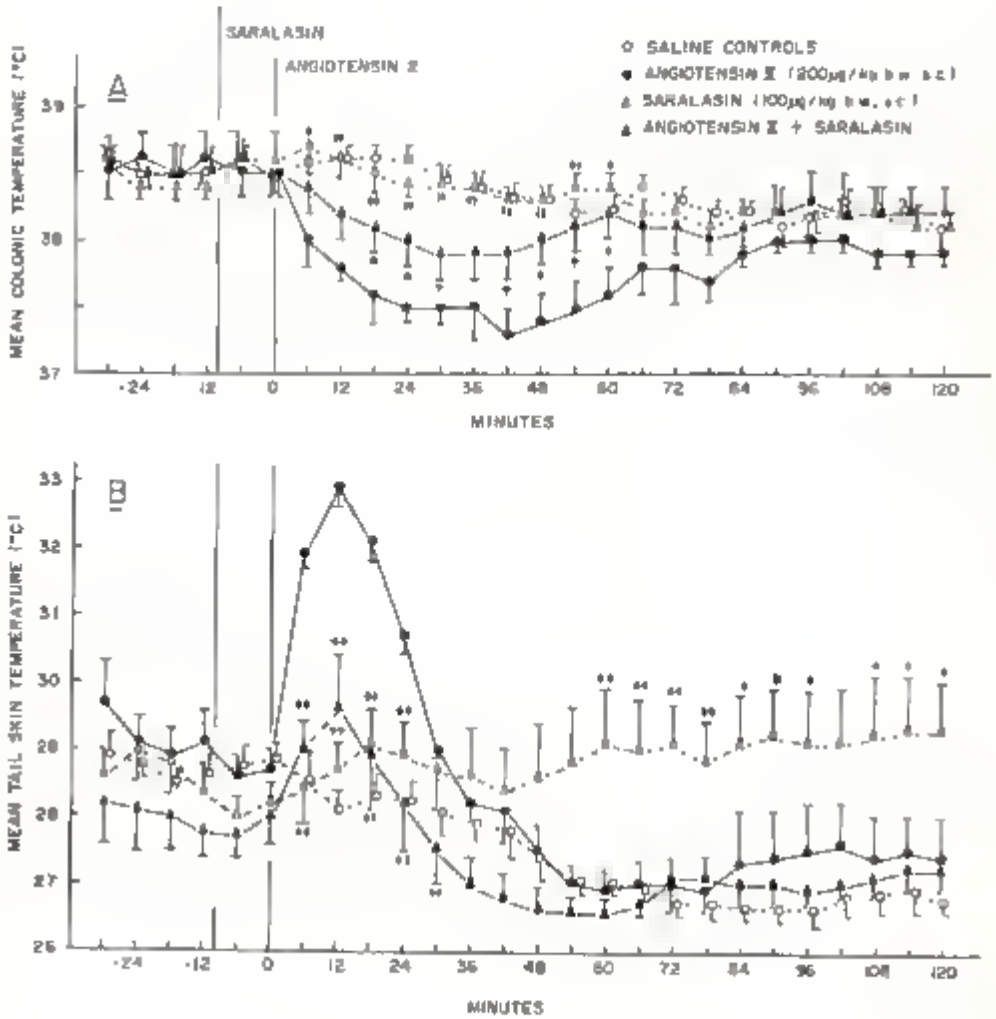

ANGIOTENSIN II (200μg/kg)
SARALASIN (1μg/kg)
SARALASIN (10μg/kg)
ANGIOTENSIN II + SARALASIN (1μg/kg)
ANGIOTENSIN II + SARALASIN (10μg/kg)
A
SARALASIN
ANGIOTENSIN II
MEAN COLONIC TEMPERATURE (°C)
40
39
38
24
12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
33
32
31
30
29
28
27
26
MINUTES

Figure 19. Effect of 100 ug saralasin/kg, s.c. on mean colonic (A) and tail skin (B) temperatures of rats administered either AII (200 ug/kg, s.c.) or saline is shown. Saralasin was administered 10 min prior AII. One SE is set off at each mean.
†Significantly different from AII ($p<0.05$).
‡Significantly different from AII ($p<0.01$).


SARALASIN
ANGIOTENSIN II
A
SALINE CONTROLS
ANGIOTENSIN II (200µg/kg b.w. s.c.)
SARALASIN (100µg/kg b.w. s.c.)
ANGIOTENSIN II + SARALASIN
MEAN COLONIC TEMPERATURE (°C)
39
38
37
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
33
32
31
30
29
28
27
26
MINUTES

administration of AII was not significantly affected by pretreatment with the lower doses (Fig. 18B). In fact, administration of AII reduced the prolonged vasodilatory effect observed during treatment with the antagonist. Administration of 100 ug saralasin/kg also produced a prolonged agonist-like increase in TST of 1.0°C, which was likewise diminished by the combined treatment. However, at this dose of saralasin, the maximal increase in TST induced by AII was reduced to 1.6°C (Fig. 19B).

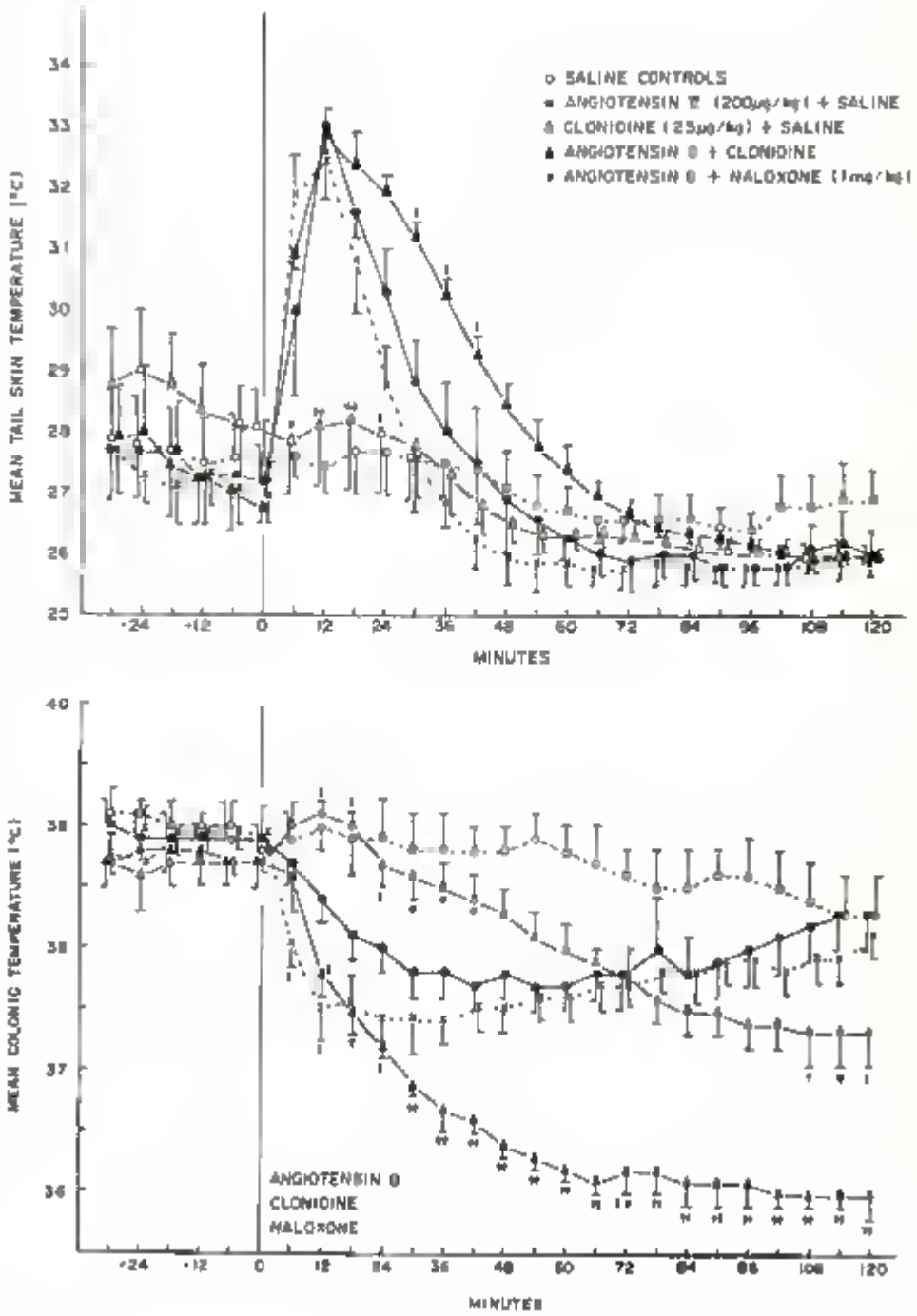
To determine whether a more complete blockade of the hypothermic response induced by AII could be achieved, the dose of AII was reduced to 100 ug/kg. Saralasin (100 ug/kg) significantly attenuated both the fall in CT and the increase in TST induced by 100 ug AII/kg; however, the extent of the reduction was not different from that seen at the higher dose.

Experiment 4A. Effect of Administration of Either Clonidine or Naloxone on the Responses of Tail Skin and Colonic Temperatures to Acute Administration of Angiotensin II

Naloxone, an opioid receptor antagonist, had no significant effect on CT when administered alone, but when combined with AII, naloxone hastened the onset of the fall in CT (Fig. 20A). Within 12 min, a decrease in CT of 1.4°C was observed in the rats treated with naloxone and AII. The AII-treated group did not attain a comparable temperature until after 24 min. Administration of the presynaptic alpha-receptor agonist, clonidine, also enhanced the reduction in CT induced by AII, but the effect was much more dramatic since clonidine alone induced a 1.5°C reduction in CT. The combined treatment of clonidine and AII induced a maximal additive decrease in CT of 2.8°C.

Neither clonidine nor naloxone altered basal TST. The AII-induced increase in TST was not affected by treatment with naloxone, while clonidine prolonged

Figure 20. Effect of either clonidine (25 ug/kg, s.c.) or naloxone (1 mg/kg, i.p.) on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. One SE is set off at each mean. †Significantly different from AII ($p<0.05$). ††Significantly different from AII ($p<0.01$).


o SALINE CONTROLS
▪ ANGIOTENSIN II (200μg/kg) + SALINE
Δ CLONIDINE (25μg/kg) + SALINE
▲ ANGIOTENSIN II + CLONIDINE
▪ ANGIOTENSIN II + NALOXONE (1mg/kg)
MEAN TAIL SKIN TEMPERATURE (°C)
34
33
32
31
30
29
28
27
26
25
-24
-12
0
12
24
36
48
60
72
84
96
108
120
MINUTES
MEAN COLONIC TEMPERATURE (°C)
40
38
38
37
36
ANGIOTENSIN II
CLONIDINE
NALOXONE
-24
-12
0
12
84
36
48
60
72
84
96
108
120
MINUTES

the vasodilation of the tail (Fig. 20B). The maximal increase of 6.1°C was not significantly different from the response induced by AII, but the TST of clonidine + AII- treated rats remained significantly elevated above both control and AII-treated groups for 18 additional min.

Experiment 4B. Effect of Administration of Yohimbine on Angiotensin II-Induced Hypothermia

Since yohimbine has been shown to enhance AII-induced dipsogenesis, the effect of this alpha$_2$-adrenoceptor antagonist on AII-induced hypothermia was evaluated. Pretreatment with yohimbine failed to affect either basal CT or the decrease in CT induced by AII (Fig. 21). With respect to TST, administration of yohimbine alone to rats induced a slow, prolonged elevation of TST (4.1°C). The addition of AII to yohimbine-treated rats evoked a maximal increase of 6.3°C, which, because of the time-course of the treatments, was significantly elevated above the response to AII alone at 6 min. Interestingly, the decrease in TST which follows the initial increase that occurs after administration of AII was also observed in the AII + yohimbine-treated group. Thus, the effect of AII appeared to override the effect of yohumbine.

Experiment 4C. Effect of Pretreatment with Indomethacin on the Responses of Tail Skin and Colonic Temperatures to Acute Administration of Angiotensin II

To determine whether the increase in TST accompanying administration of AII was due to the mediation of prostaglandins, indomethacin, a cyclooxygenase enzyme inhibitor, was administered prior to AII (Fig. 22). Pretreatment with indomethacin augmented the fall in CT at both doses used, but did not significantly affect the elevation in TST.

Figure 21. Effect of yohimbine on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Yohimbine (300 ug/kg, s.c.) was administered 15 min prior to AII (200 ug/kg, s.c.). One SE is set off at each mean.
†Significantly different from AII ($p<0.05$).
‡Significantly different from AII ($p<0.01$).

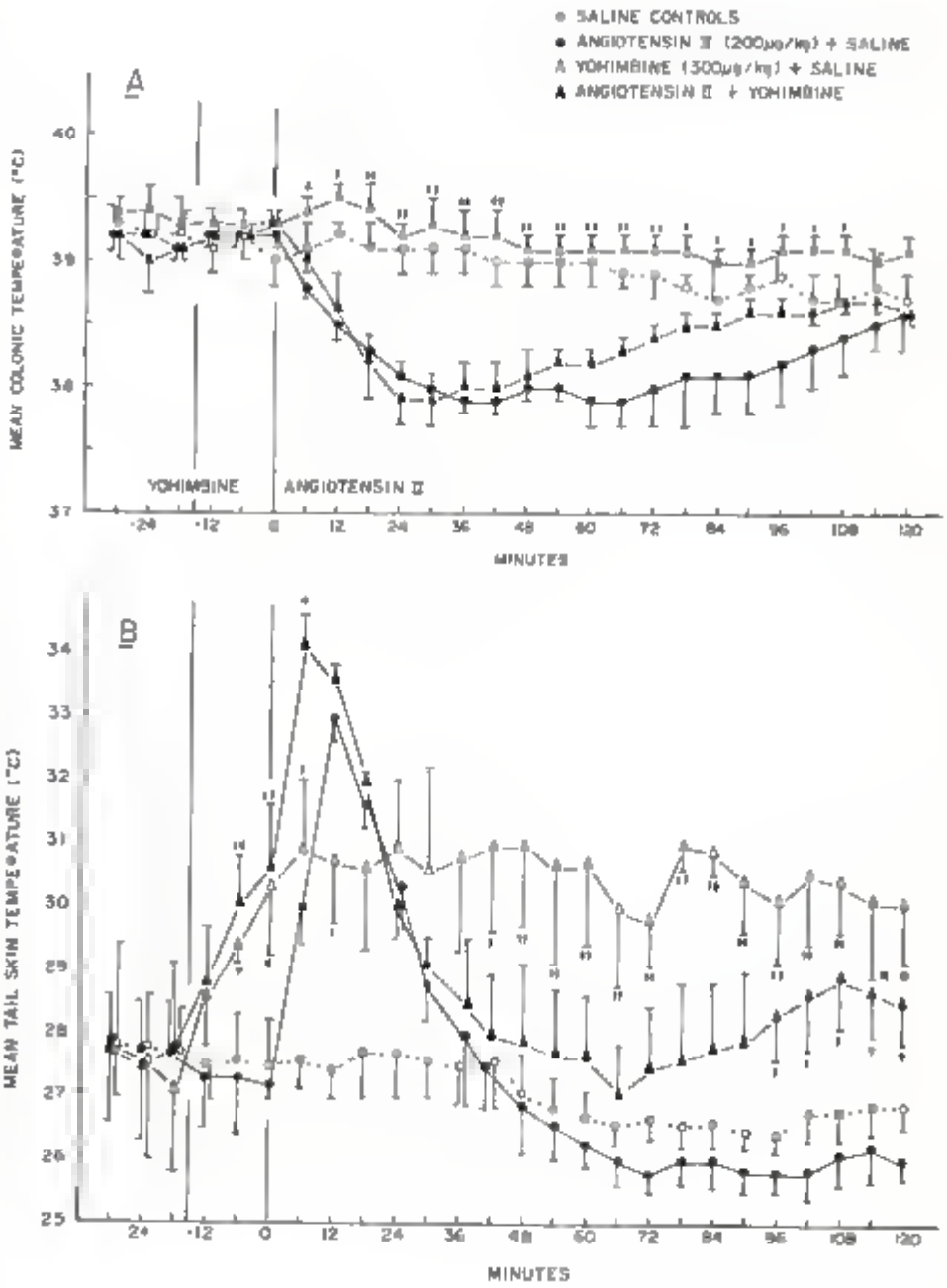
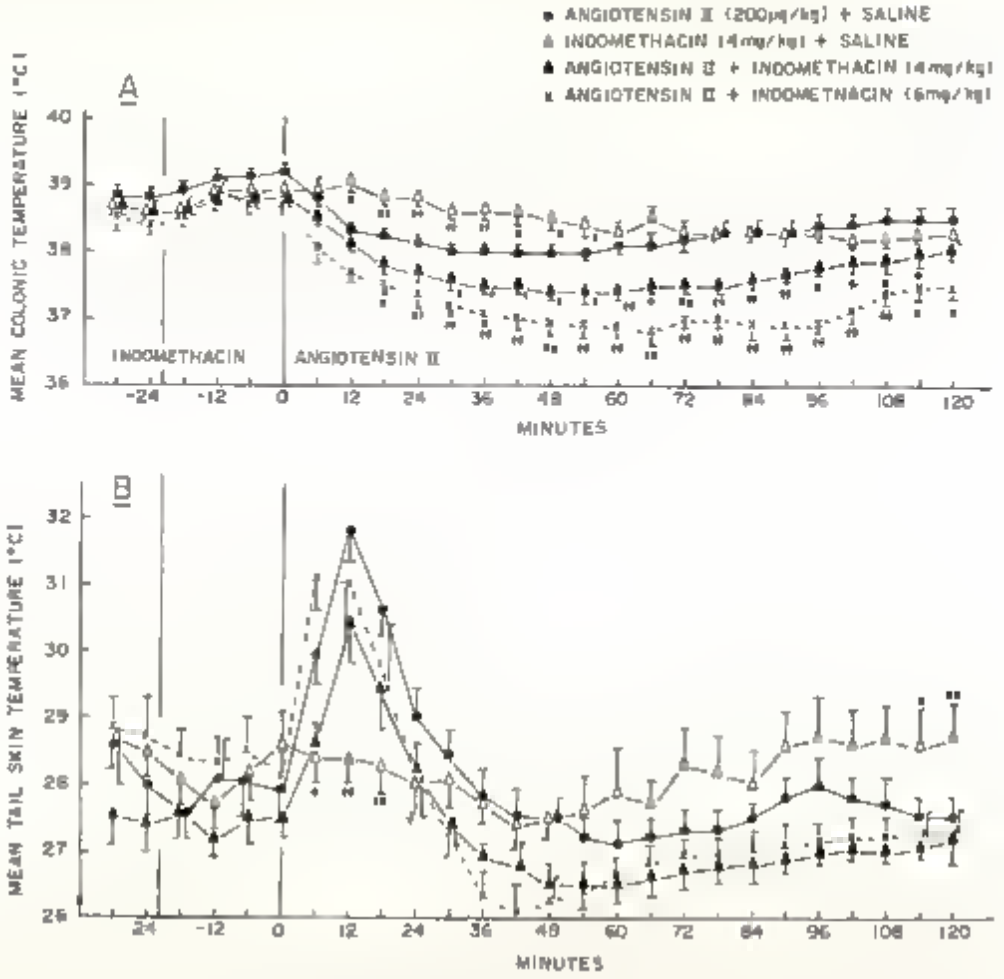

SALINE CONTROLS
ANGIOTENSIN II (200µg/kg) + SALINE
YOHIMBINE (300µg/kg) + SALINE
ANGIOTENSIN II + YOHIMBINE
A
MEAN COLONIC TEMPERATURE (°C)
YOHIMBINE
ANGIOTENSIN II
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
MINUTES

Figure 22. Effect of indomethacin on AII-induced hypothermia. Mean colonic (A) and tail skin (B) temperatures are shown. Indomethacin (either 4 or 6 mg/kg, s.c.) was administered 20 min prior to AII (200 ug/kg, s.c.). One SE is set off at each mean. †Significantly different from AII ($p<0.05$). ‡Significantly different from AII ($p<0.01$).


ANGIOTENSIN II (200μg/kg) + SALINE
INDOMETHACIN 14mg/kg) + SALINE
ANGIOTENSIN II + INDOMETHACIN 14mg/kg)
ANGIOTENSIN II + INDOMETHACIN (6mg/kg)
A
MEAN COLONIC TEMPERATURE (°C)
INDOMETHACIN
ANGIOTENSIN II
MINUTES
B
MEAN TAIL SKIN TEMPERATURE (°C)
MINUTES

Discussion

The hypothermic response elicited by subcutaneous administration of AII appears to be: (a) specific for the octapeptide; (b) inducible with central administration of AII; and (c) mediated by mechanisms distinct from AII-induced pressor and dipsogenic responses. As previously demonstrated, subcutaneous administration of AII produced a fall in CT of 1.3°C and an increase in TST of 4.2°C. Angiotensin I produced an identical response which was inhibited when the conversion of AI to AII was prevented with the converting enzyme inhibitor, captopril. In contrast, the heptapeptide, AIII, which is proposed to mediate several of the effects of AII, did not influence thermoregulatory responses. Thus, the degradation of AII to AIII does not appear necessary for the hypothermic responses accompanying administration of AII. The specificity of the response is reinforced by the ability of saralasin, a specific AII-receptor antagonist, to reverse partially both the increase in TST and the fall in CT. Therefore, the hypothermic response which accompanies administration of AII is mediated by AII receptors, the location of which is uncertain but could be within the central nervous system.

Although the metabolic effect of central administration of AII has been investigated, the results of these investigations are not consistent due both to the dose and route of administration (i.v.t. vs microinjections into specific brain regions) (Kiyohara et al., 1984; Lin et al., 1980; Sharpe et al., 1979). In the present study, i.v.t. administration of AII did not appear to induce as great a hypothermia as peripheral administration. Colonic temperature fell only 0.4°C, one-third of the decrease observed with the peripheral response. The effect of central administration of AII on TST was about half that induced by peripheral administration (2.4°C). This reduction in central responsiveness contrasts with the participation of the

brain angiotensin system in pressor and dipsogenic responses. The threshold for drinking to intracranial administration of AII in the water-replete rat is 10^{-16} to 10^{-15} mol. At a dose of 100 ng (10^{-10} mol.), which did not affect temperature, the amount drunk in 15 min approached the total amount of water a rat would consume in 24 hr (Simpson et al., 1978). As little as 50 fg, administered directly into the optic recess, can also elicit pressor responses (Phillips, 1976). Thus, about 10^6 fold more AII is required to produce a hypothermic response in the rat by i.v.t. administration than that required for dipsogenic and pressor responses. The time-course of the responses also differs. Both the pressor and dipsogenic responses occur within one min and are completed within about 15 min. As is observed in Figure 16, the effect of i.v.t. administration of AII is not fully apparent until 15 min after injection. The dipsogenic effect of peripheral administration of AII is also somewhat delayed when compared to the central response, while the reverse occurs in temperature responsiveness. It is possible that the i.v.t. route of administration may not afford ready accessibility to the site of action. If this is the case, it would suggest that the locus for the mediation of AII-induced hypothermia is different from that for AII-induced dipsogenic and pressor responses.

Some insight into the location of a central site mediating the hypothermic response to AII has been given by Kiyohara et al. (1984) who reported that bilateral microinjections (0.2-1.0 ug) into the medial preoptic area (MPO) of rats could elicit transient decreases in CT which correlated with increased activity of iontophoretically stimulated warm unit neurons in the MPO. In addition, Sharpe et al. (1979) indicated that microinjections of 1.5 ug AII into the lateral regions of the forebrain and medial regions in the hypothalamus, thalamus, midbrain and pons could elicit a 0.5°C fall in CT

in the rabbit. Activation of thermosensitive neurons located in the MPO are proposed to mediate vasodilatory and other heat-activated responses which suggests that AII may initiate directly the thermoregulatory responses (Hensel, 1973). Regions which surround the MPO, such as the antero-ventral third ventricle, subfornical organ and the organum vasculosum of the lamina terminalis, mediate the dipsogenic and pressor responses to AII in rats (Lind and Johnson, 1982). However, it has been demonstrated that lesions in the MPO region of the rat brain do not inhibit the drinking responses to either central or peripheral administration of AII (Kucharczyk et al., 1976). Lateral regions of the forebrain are likewise not known to mediate either the dipsogenic or pressor effects of AII. Since the doses required for a central thermoregulatory response are so large, it appears that diffusion from the microinjected areas may be required.

Several of the other facets of AII-induced hypothermia suggest that this effect is mediated by mechanisms different from those mediating its pressor and dipsogenic responses. Both peripheral and i.v.t. administration of AIII have also been shown to increase blood pressure and water intake in rats (Fitzsimons, 1971; Tonnaer et al., 1982; Wright et al., 1984). It is proposed that degradation of AII to AIII accounts for a portion of the dipsogenic and pressor effects of AII. The inability of AIII to mimic the hypothermic effect of AII suggests that the response is specific for AII.

Additional studies using saralasin support the suggestion that AII-induced hypothermia is a specific receptor-mediated event. The agonistic effect of saralasin observed with each of the doses is also apparent in the other AII-induced effects. This system, however, appears to be more sensitive to inhibition than either the dipsogenic or pressor responses since the hypothermic responses were attenuated by a dose of saralasin half that of AII. For central blockade of the drinking response to AII, a 10:1 ratio of

saralasin:AII is required (Epstein et al., 1974). To inhibit the pressor response to an acute i.v. infusion of AII, the amount of saralasin necessary is 5-7 times the amount of AII (Mann et al., 1983).

The role of catecholamines in the regulation of blood pressure and drinking is well documented: AII has been shown to release norepinephrine (NE) from sympathetic nerve endings (Hughes and Roth, 1971); and either depletion of central catecholamines with 6-hydroxydopamine or stimulation of central alpha$_2$-adrenoceptors, which inhibits NE release, significantly diminishes the dipsogenic and pressor responses to acute administration of AII (Fitzsimons and Setler, 1975; Fregly et al., 1984a,b; Gordon et al., 1979). In addition, NE is proposed to play a role in temperature regulation; however, different effects are obtained depending upon the dose and route of administration (Veale and Whishaw, 1976). The administration of NE into the preoptic/anterior hypothalamic area has been shown to evoke a reduction in CT and an increase in TST (Poole and Stephenson, 1979). The inability of the alpha$_2$-adrenoceptor agonist, clonidine, to antagonize the hypothermic effect of AII reinforces a dissociation of its hypothermic effect from its pressor and drinking effects.

The effect of naloxone on drinking behavior is often interpreted as an indication of the involvement of opioids in ingestive behaviors (Brown and Holtzman, 1981b). However, as discussed previously, the effect of naloxone on AII-induced dipsogenesis may be due to its central inhibition of NE release. In either case, the administration of naloxone did not reduce the hypothermic effect of angiotensin. Naloxone has been reported to block the hypothermic effects of apomorphine, chlorpromazine and ethanol in mice (Weiss et al., 1984). This suggests that opioids are not involved in AII-induced hypothermia.

Yohimbine, an alpha$_2$-adrenoceptor antagonist, can reverse the dipsogenic inhibition of clonidine and naloxone, as well as the hypothermic

effect of clonidine (Yehuda, 1975). Yohimbine also potentiates the drinking behavior induced by AII (Fregly et al., 1983a). Although not as dramatic as clonidine, yohimbine also enhanced the hypothermia accompanying administration of AII. Thus, antagonism by either alpha- or beta-adrenergic antagonists did not attenuate the AII-induced hypothermia which suggests that NE probably does not mediate the response.

The versatility of the trophic activity of angiotensin includes its ability to stimulate production of prostaglandins in vascular tissue (Blumberg et al., 1977). In addition, prostaglandins are released in response to pressor stimuli and serve to antagonize the pressor effects with their potent vasodilating properties (Zawada, 1982). On the assumption that the vasodilation of the tail of the rat may be due to the production of prostaglandins, the cyclooxygenase enzyme inhibitor, indomethacin, was administered. Once again, pharmacological intervention was without effect. Prostaglandins probably are not involved in the vasodilatory effect of AII.

The inability to reverse the increase in TST with pharmacological treatments known to antagonize other responses to angiotensin suggests that the vasodilation may be due to the intrinsic properties of the tail. It has been demonstrated that tail blood flow can be regulated either as a function of colonic temperature or as a function of tail temperature and independent of central body temperature (Raman et al., 1983). As temperature is increased above 37°C, blood flow and heat flow to the tail also increase. In this respect, the thermal control of the blood flow in the tail of the rat resembles that of the human forearm. However, in AII-induced temperature responses, CT decreases concomitantly with an increase in TST. Thus, the rat responds to administration of AII as if it were either being subjected to an acute heat

load or its internal thermoregulatory controls were readjusted to operate at a lower core temperature. Indeed, it has been reported that plasma renin activity (PRA) increases when humans are subjected to a heat stress for 40-50 min (Escourrou et al., 1982). This increase in PRA can be blocked by administration of propranolol, a beta-adrenoceptor antagonist which blocks the release of renin from the kidneys. Blood flow to the forearm also increased during heat exposure and this response was likewise attenuated by propranolol. Although the inhibitory effect on forearm blood flow was attributed to the decrease in blood pressure by propranolol, the possibility that the reduction in PRA may have contributed to the response should not be disregarded. Clonidine also reduces blood pressure, but the vasodilatory effect of AII and presumed increase in blood flow persisted. Thus, the possibility exists that AII may be intricately involved in temperature regulation, in particular, during heat stress. The fall in colonic temperature appears to be mediated through a central cholinergic system, and the activation of heat loss mechanisms may involve the direct effect of AII upon the vasculature of the tail. Whether this is due to a receptor-mediated vasodilation or an alteration in the blood flow within the tail has yet to be established.

CHAPTER VI
MINERALOCORTICOIDS MODULATE CENTRAL ANGIOTENSIN II RECEPTORS IN RATS

Introduction

Whether administered peripherally or centrally, the octapeptide, angiotensin II (AII), is believed to interact with its receptors in the central nervous system to elicit drinking and pressor responses (Fitzsimons, 1980; Lind and Johnson, 1982; Mann et al., 1981; Phillips, 1980; Severs et al., 1971; Sirett et al., 1979). The physiological factors which regulate central AII receptors are unclear at present. However, there is some evidence that steroid hormones may influence the binding of AII to its receptors in both peripheral tissues and in the central nervous system. Thus, binding of AII to its receptors in mesenteric arteries and adrenal cortices of the rat is influenced both by the presence in excess and by the absence of mineralcorticoid hormones (Douglas and Brown, 1982; Schiffrin et al., 1984; Schiffrin et al., 1983b). Further, additional studies have indicated that chronic administration of estradiol reduces the binding of AII to its receptors in the hypothalamus (Fregly et al., 1985; Jonklaas and Buggy, 1985). The present studies were carried out to determine whether chronic administration of deoxycorticosterone acetate (DOCA) to rats affected the binding of AII to its receptors in a block of tissue containing the hypothalamus, thalamus and septum (HTS) and to assess whether these changes were manifested in the dipsogenic and pressor responsiveness to both peripheral and central administration of AII.

Chronic administration of DOCA has been shown to deplete the kidneys of renin and thereby reduce plasma renin activity and circulating levels of AII (Tobian, 1959; Tobian, 1960). Since the concentration of AII in plasma can regulate its own receptors in the adrenal, blood vessels, uterus and bladder (Aguilera et al., 1978; Catt et al., 1984; Devynck et al., 1976; Douglas and Brown, 1982; Gunther et al., 1980; Hauger et al., 1978), the possibility existed that changes in the binding of AII to its receptors in the HTS of rats treated chronically with DOCA might be a reflection of the plasma level of AII. Therefore, additional studies were carried out using neuronal cell cultures prepared from the brains of one-day-old rats to study the effect of mineralocorticoid hormones on the binding of AII to its receptors in cultured neurons. Cell cultures were used to minimize the possible effects of circulating AII and to determine whether the steroid action was either direct or indirect.

Methods

Experimental Protocol

A) Thirty-two female rats weighing 200-260 g were used. They were kept 3 per cage in a room maintained at 26 ± 1°C and illuminated from 0700 to 1900. All rats were provided with Purina Laboratory Chow and 0.9% NaCl to drink. Half of the rats was anesthetized with ether, and Silastic tubes (#602-235) containing DOCA were implanted subcutaneously between the shoulder blades. The other half was implanted with empty Silastic tubes. Prior to implantation and upon removal after the experiment, the 21 mm tubes were placed in a vaccuum dessicator for 72 hr and then weighed on an analytical balance. Daily drug dose was calculated from the weight loss of each tube divided by the number of days the tubes were implanted. Dimethylpolysiloxane (Silastic) tubing has been

shown to allow diffusion of certain crystalline steroids into various media at a constant rate over relatively long peroids of time (Dzuik and Cook, 1966; Kincl et al., 1968).

After five weeks of treatment with DOCA, the drinking response to acute administration of AII (200 ug/kg, s.c.) was tested. These rats then received intracerebroventricular cannulae to test their drinking responses to central administration of AII (10 ng). Two weeks later, rats were killed by guillotine decapitation. Blood was collected in EDTA and centrifuged at 4°C. Plasma was separated and stored at 4°C for determination of plasma concentration of aldosterone and plasma renin activity by radioimmunoassay. The brain was removed and the region containing the hypothalamus, thalamus, and septum was dissected free for AII-receptor binding studies as described in the general methods.

B) Twenty-four female rats were used to study the pressor responsiveness to peripherally and centrally administered AII in DOCA-treated rats. DOCA was administered either as described above (240 ug/kg/day via Silastic tubes) or by an additional weekly subcutaneous treatment of 12.5 mg of DOC pivalate/kg. DOCA-treated rats and controls were given isotonic saline to drink. Treatments with DOCA failed to induce an elevation of blood pressure in any of the groups.

After 8 to 10 weeks of treatment with DOCA, rats were anesthetized with ketamine hydrochloride (130 mg/kg) and acepromazine (50 ug/kg) while catheters were implanted. Polyethylene tubing (PE 50) was filled with 0.9% saline containing 50 U heparin/ml, inserted into the femoral artery and secured. The free end of the catheter was passed under the skin and brought out at the scruff of the neck. The arterial catheter was connected to a Statham transducer which was coupled to a polygraph for continous monitoring of blood pressure. Pressures were allowed to stabilize and AII was administered either

s.c. or i.v.t. to each anesthetized rat. Blood pressures were then recorded until pressures returned to pre-injection level. Two days later, the treatments were reversed and pressor responses were again recorded, but in this case, the rats were conscious and freely moving. The s.c. dose of AII was either 10 ug/kg or 20 ug/kg and the centrally administered dose was 25 ng. Mean blood pressure was calculated as 1/3 pulse pressure + diastolic pressure.

Preparation of Neuronal Primary Brain Cell Cultures

Neuronal primary cultures were prepared from 1-day-old rat brains essentially as described previously (Raizada et al., 1982; Summers et al., 1983). Brains were removed and placed in an isotonic salt solution containing 100 units of penicillin G, 100 ug streptomycin and 0.25 ug/ml of amphotericin B (Fungizone), pH 7.2. All pia mater and blood vessels were removed from the brains, which were then chopped into approximately 2 mm chunks. The minced tissue was suspended in 25 ml 0.25% (wt/vol) trypsin in isotonic salt solution and placed for 7 min at 37°C in a shaking water bath. Tissue pieces sedimented and the dissociated cells were collected and mixed with 10 ml of Dulbecco's modified Eagles medium (DMEM) containing 10% plasma-derived horse serum (PDHS). Undissociated tissue was agitated, treated with 160 ug deoxyribonuclease I, and returned to the shaker bath for an additional 6 min. The cells were washed with 40 ml DMEM containing PDHS, and centrifuged at 1000 x g for 10 min. Recoveries were normally 40-50 x 10^6 cells/g of tissue. Cells (3.0 x 10^5) were resuspended in DMEM containing 10% PDHS and plated in Falcon tissue culture dishes (35 mm diameter) precoated with poly-L-lysine for the AII binding experiments. Cells were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air. After 3 days of incubation, cells were treated with 10 uM cytosine arabinoside (ARC) prepared in DMEM

containing 10% PDHS. This treatment inhibits the multiplication of cells, the majority of which are non-neuronal in origin and results in neuronal cultures. After 48 hrs, the ARC-medium was replaced with fresh DMEM containing 10% PDHS. Cells were incubated for an additional 6-10 days before being used for binding experiments.

Mineralocorticoid Treatment of Neuronal Cell Cultures

After 10 days in growth medium, the cells were treated with either deoxycorticosterone acetate (DOCA) or aldosterone (ALDO). DOCA and ALDO were dissolved in 95% ethanol and diluted in PBS. Twenty - 100 ul of the final dilution was added to the two ml of medium. The mineralocorticoids were added at either 30, 20, 15 or 5 hr prior to experimentation and the doses ranged from 1 to 10,000 pg/ml (2 to 28,000 pM).

Receptor Binding Assay in Culture

The specific binding of [^{125}I]-AII to cell membrane receptors was determined in intact brain cells attached to culture dishes (35 mm diameter). Growth medium was removed and cells were washed three times with Dulbecco's phosphate-buffered saline (PBS), pH 7.2. Unless otherwise specified, triplicate cultures were incubated for 30-60 min at 24^{o}C with 500 ul PBS containing 0.2 nM [^{125}I]-AII (150,000 cpm) and 0.8% BSA. Additional cultures were incubated in triplicate for 30-60 min at 24^{o}C with the same reaction mixture containing 150 nM unlabelled AII. Following the incubation period, cells were rinsed rapidly three times with ice cold PBS containing 0.8% BSA. Cells were dissolved with 500 ul 2.0 N NaOH and transferred to plastic tubes with the aid of a rubber policeman. The culture dish was then rinsed with 500 ul distilled water which was combined with the original sample. Radioactivity was determined in a Beckman gamma counter at a counting efficiency of 75% for ^{125}I. Specific binding of [^{125}I]-AII to cell

membrane receptors was determined from the mean of triplicate samples obtained by subtracting radioactivity bound in the presence of unlabeled AII from the total radioactivity bound. Results are expressed as means ± one standard error. Statistical significance was calculated by one-way analysis of variance, and Student's paired and unpaired t-tests. Regression lines were calculated by method of least squares (Zar, 1974).

Protein Contents and Determination of Cell Number

The protein content per culture dish was determined by assaying 100 ul of the dissolved cells using the method of Lowry et al. (1951). The number of cells per plate was calculated as follows. Growth media were aspirated and cells were washed three times with PBS (pH 7.2). Cells were then dissociated with 1 ml 0.25% trypsin (wt/vol) and added to 1 ml DMEM containing 10% FDHS. A drop of this suspension was placed on a hemocytometer, and cells were counted using an inverted microscope (Leitz).

Results

Dipsogenic and Blood Pressure Responses

Acute s.c. administration of AII induced a significantly greater (45%) drinking response in rats chronically treated with DOCA than in controls at all times after treatment. The drinking response to i.v.t. administration of AII also was increased significantly (80%) in DOCA-treated rats at each of these time intervals (Fig. 23).

Treatment with DOCA at either 240 ug/kg/day or 2 mg/kg/day did not significantly alter basal mean blood pressure (BP) of conscious rats (Fig. 24). Likewise, the changes induced by either central or peripheral administration of AII were not different between the two DOCA treatments, so the data from both were combined. Administration of ketamine significantly lowered the BP of

Figure 23. Dipsogenic effect of subcutaneous administration of AII (200 ug/kg, s.c.) (left panel) to control (open bar) or DOCA-treated (240 ug/kg/day) (hatched bar) rats at 0.5, 1.0, and 2.0 hr after treatment. The dipsogenic response to i.v.t. (10 ng) administration of AII to the same group of rats is shown in the right panel. One SE is set off at each mean (n = 8). *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

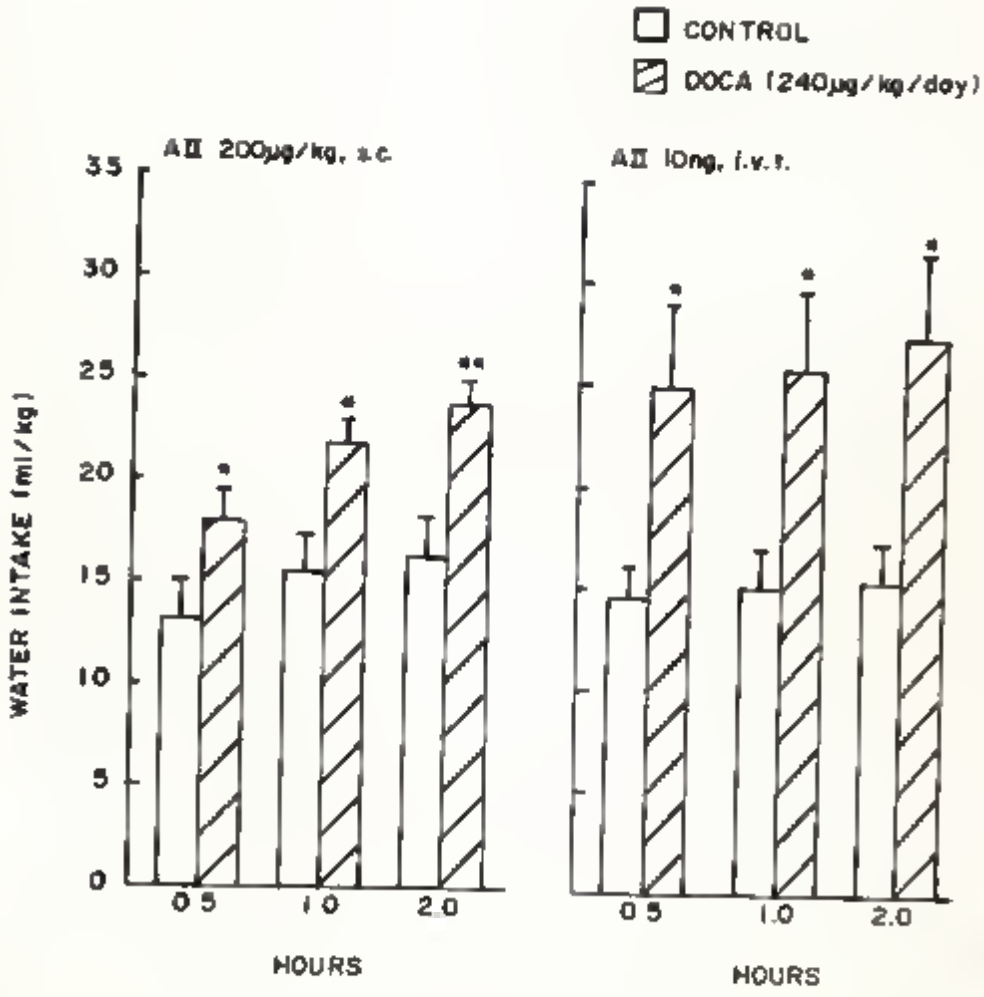
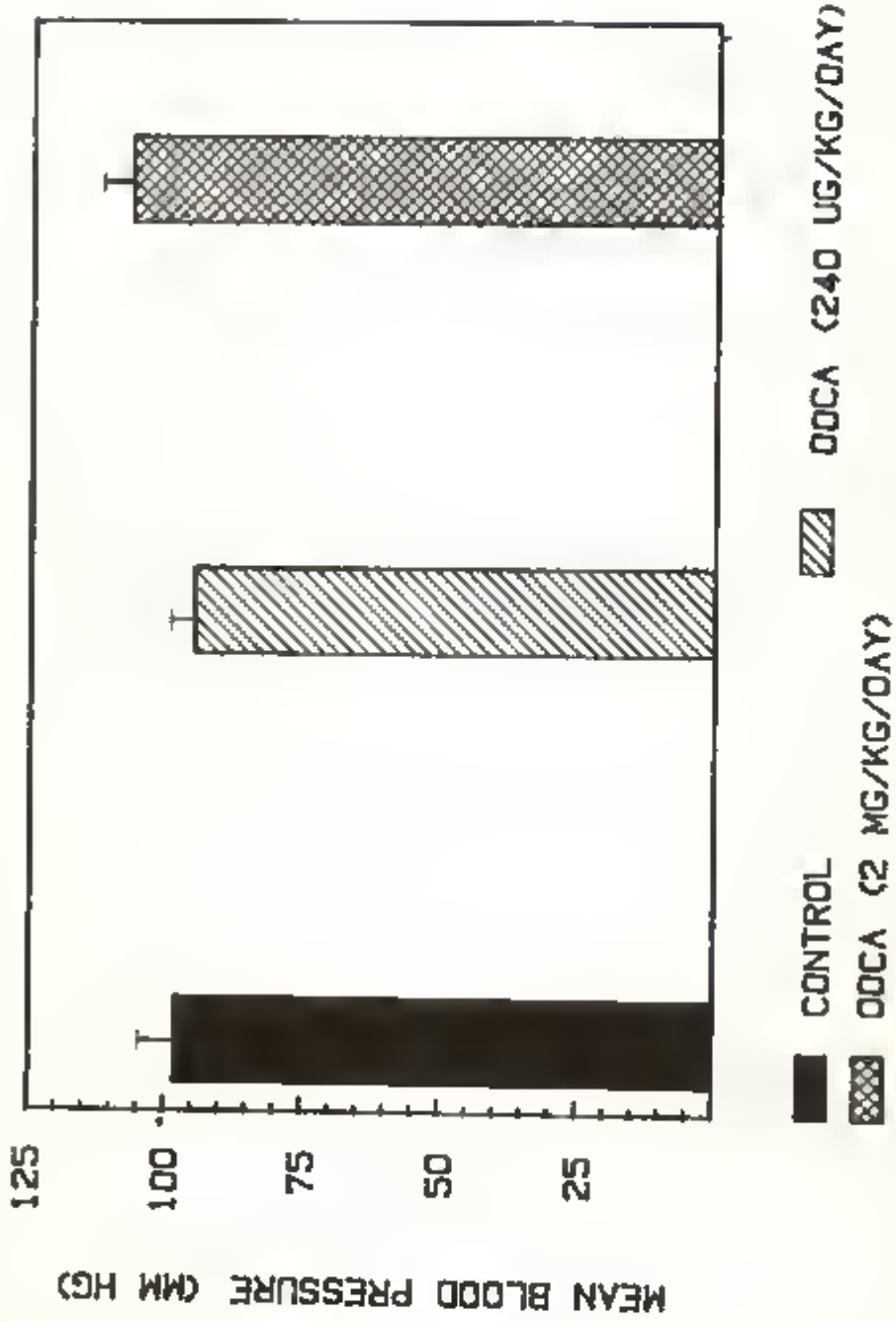

CONTROL
DOCA (240µg/kg/day)
AII 200µg/kg, s.c.
AII 10ng, i.v.t.
WATER INTAKE (ml/kg)
35
30
25
20
15
10
5
0
0.5
1.0
2.0
HOURS
*
**

Figure 24. Basal mean blood pressures in conscious rats. Controls are compared to rats treated chronically with either 240 ug/kg/day or 2 mg/kg/day of DOCA. One SE is set off at each mean (n = 16). There are no significant differences between the groups.


MEAN BLOOD PRESSURE (MM HG)
125
100
75
50
25
CONTROL
DOCA (2 MG/KG/DAY)
DOCA (240 UG/KG/DAY)

DOCA-treated rats, but not that of controls (Table 3). However, the changes in BP induced by administration of AII were similar in both anesthetized and awake animals. Intraventricular administration of AII elicited a pressor response of significantly greater magnitude in both the anesthetized and conscious DOCA-treated rats than in their controls ($p<0.01$ and $p<0.05$: Table 3). Subcutaneous administration of 10 ug AII/kg increased BP in both anesthetized and conscious control and DOCA-treated rats, but the difference between groups was not significant. However, the change in mean BP in the anesthetized DOCA-treated group to s.c. administration of 20 ug AII/kg was significantly elevated above that of controls ($p<0.05$).

Angiotensin II-binding to the HTS Region of the Brain

The binding of AII to membranes prepared from the HTS of individual control and DOCA-treated rats was performed with both 0.25 and 1.0 nM [^{125}I]-AII in the incubation medium. In DOCA-treated rats, the specific binding of [^{125}I]-AII to brain membranes was significantly increased compared with controls (Fig. 25). This increase was 29% at 0.25 nM and 73% at 1.0 nM [^{125}I]-AII in the incubation medium.

To determine whether the increase in binding was due to changes in either the number or the affinity of AII binding sites, the specific binding of [^{125}I]-AII to brain tissue from DOCA-treated and control rats was studied as a function of AII concentration. Figure 26 shows a representative saturation experiment. Each point represents the specific binding values of pooled tissue from 4 rats. After a 45 min incubation, the specific binding reached a plateau between 0.6 and 1.0 nM concentration of AII in both the control and DOCA-treated rats. Specific binding to the brain receptor sites in DOCA-treated rats was markedly higher at concentrations of AII above 0.2 nM. Scatchard analysis (Scatchard, 1949) of these data (Fig. 26, upper panel)

TABLE 3

EFFECT OF INTRAVENTRICULAR (i.v.t.) AND SUBCUTANEOUS (s.c.) ADMINISTRATION OF ANGIOTENSIN II ON THE INCREASE IN MEAN BLOOD PRESSURE OF BOTH ANESTHETIZED AND CONSCIOUS DOCA-TREATED AND CONTROL RATS.

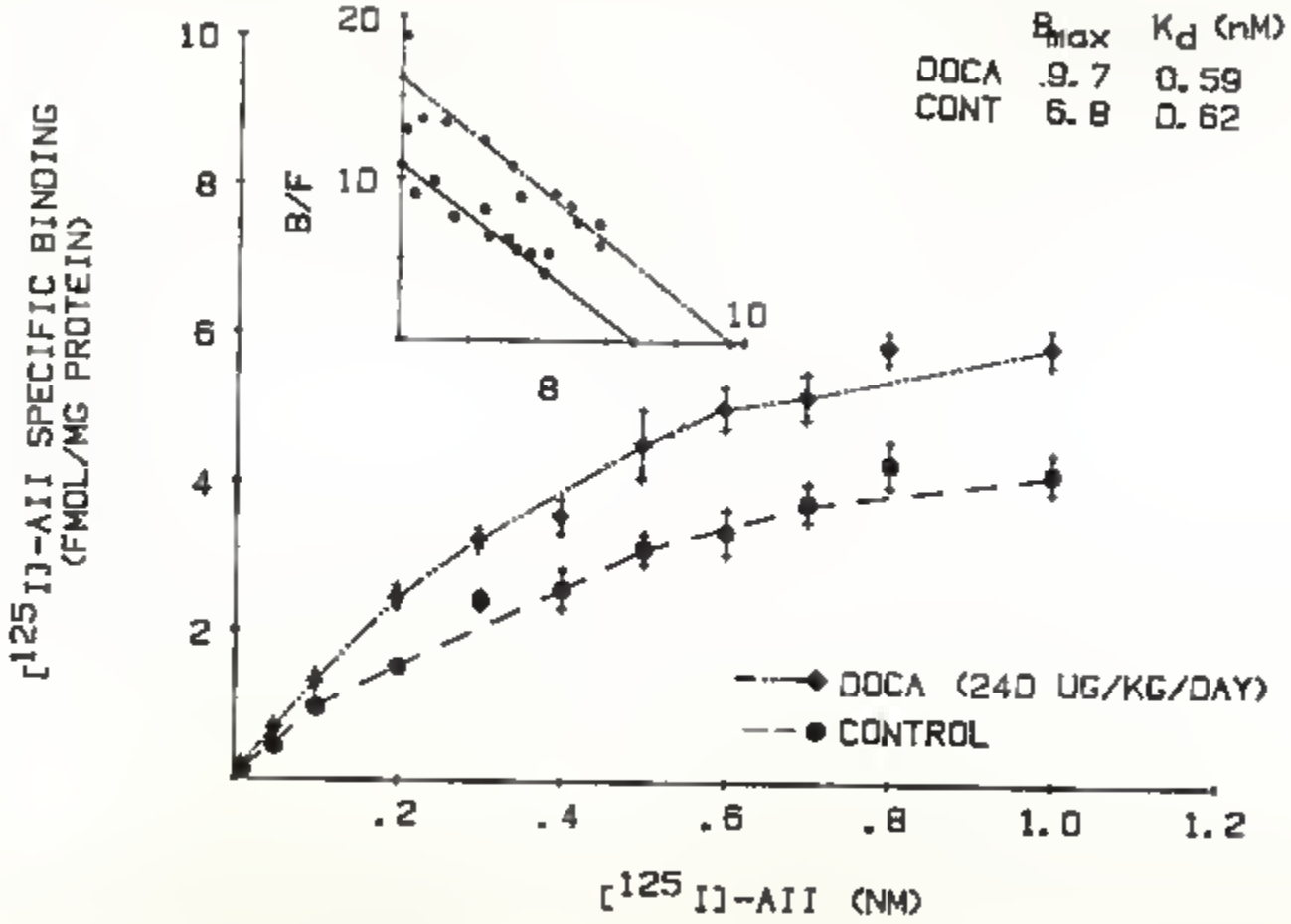
		INCREASE IN MEAN BLOOD PRESSURE (mm Hg) AFTER ADMINISTRATION OF AII		
	BASAL	i.v.t. (25 ng)	s.c. (10 ug/kg)	s.c. (20 ug/kg)
ANESTHETIZED				
CONTROL	95.9 ± 4.0+	18.0 ± 2.6	37.0 ± 5.0	42.8 ± 6.7
DOCA	82.0 ± 4.1*	32.7 ± 3.5**	39.0 ± 4.0	58.3 ± 3.3*
AWAKE				
CONTROL	98.3 ± 6.0	20.7 ± 1.8	33.7 ± 6.3	---
DOCA	102.7 ± 3.4	30.5 ± 3.2*	26.7 ± 4.2	---

+ One standard error of mean
* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)
--- No determination

Figure 25. Effect of chronic administration of DOCA on the specific [^{125}I]-AII binding at 0.25 and 1.0 nM concentrations of [^{125}I]-AII to membranes prepared from the hypothalamus, thalamus and septum (HTS) of DOCA-treated and control rats. The y-axis represents AII specific binding, and each bar is the average of individual binding results (n = 4). One SE is set off at each mean. *Significantly different from control ($p<0.05$) **Significantly different from control ($p<0.01$).

[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
12
10
8
6
4
2
.25 NM
1.0 NM
CONTROL
OOCA-SALT (240 UG/KG/DAY)

Figure 26. The effect of increasing concentrations of [^{125}I]-AII on the specific binding of [^{125}I]-AII to membranes prepared from the hypothalamus, thalamus and septum (HTS) of control rats and rats treated with DOCA (240 ug/kg/day). The lower panel represents saturation isotherms for [^{125}I]-AII specific binding in membranes prepared from DOCA-treated and control rats (n = 4). One SE is set off at each mean. The upper panel illustrates a Scatchard analysis of the same data. B (fmol/mg protein) is the specific binding at each concentration of [^{125}I]-AII, and F is the free concentration (nM) of [^{125}I]-AII.


B_{max} K_d (nM)
DOCA 9.7 0.59
CONT 6.8 0.62
B/F
20
10
10
8
[125 I]-AII SPECIFIC BINDING (FMOL/MG PROTEIN)
10
8
6
4
2
DOCA (240 UG/KG/DAY)
CONTROL
.2
.4
.6
.8
1.0
1.2
[125 I]-AII (NM)

revealed a higher maximal number of binding sites (B_{max}) in hypothalamic tissue of DOCA-treated rats (9.65 fmol/mg protein) compared with controls (6.80 fmol/mg protein). The dissociation constants (K_d) in control and DOCA-treated rats were 0.62 and 0.59 nM respectively, and were not significantly different. Additional saturation experiments showed similar increases in B_{max} in DOCA-treated rats compared with controls, and no significant changes in K_d.

Effects of Mineralocorticoids on Angiotensin II Binding in Neuronal Cell Cultures

Neuronal projections are produced by trypsin-dissociated cells from one day old rat brains within 48 hrs of being plated in poly-L-lysine-coated culture dishes. Approximately 80% of the cells remaining after treatment with cytosine arabinoside and cultured for 7 to 21 days have been shown to be of neuronal origin and contain extensive processes as indicated by previous immunocytochemical and morphological techniques (Raizada et al., 1982; Raizada et al., 1981). Specific AII binding sites have been localized by autoradiography and characterized on rat brain neurons in culture (Raizada et al., 1981; Stamler et al., 1981). Treatment with either DOCA or ALDO did not affect the number of cells per plate. Nonspecific binding did not exceed 10% of total binding in any of the experiments.

Treatment of neuronal brain cell cultures with either DOCA or ALDO elicited a time- and dose-dependent increase in the specific binding of [^{125}I]-AII to neuronal binding sites (Figs. 27 & 28). Initial studies with 1300-1400 pM of either ALDO or DOCA indicated a significant increase of 52 and 63%, respectively, in [^{125}I]-AII binding to cultured neurons at 15 hr. Treatment with aldosterone also elicited an increase at 5 hr (Fig. 27). Further studies indicated that the largest increase in binding was observed after 20 hr incubation with both of the mineralocorticoids (Not shown).

Figure 27. Specific [^{125}I]-AII (0.25 nM) binding in neuronal cell cultures prepared from the brains of one-day old rats following treatment with either ALDO (1344 pM) or DOCA (1385 pM) for different time periods. One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

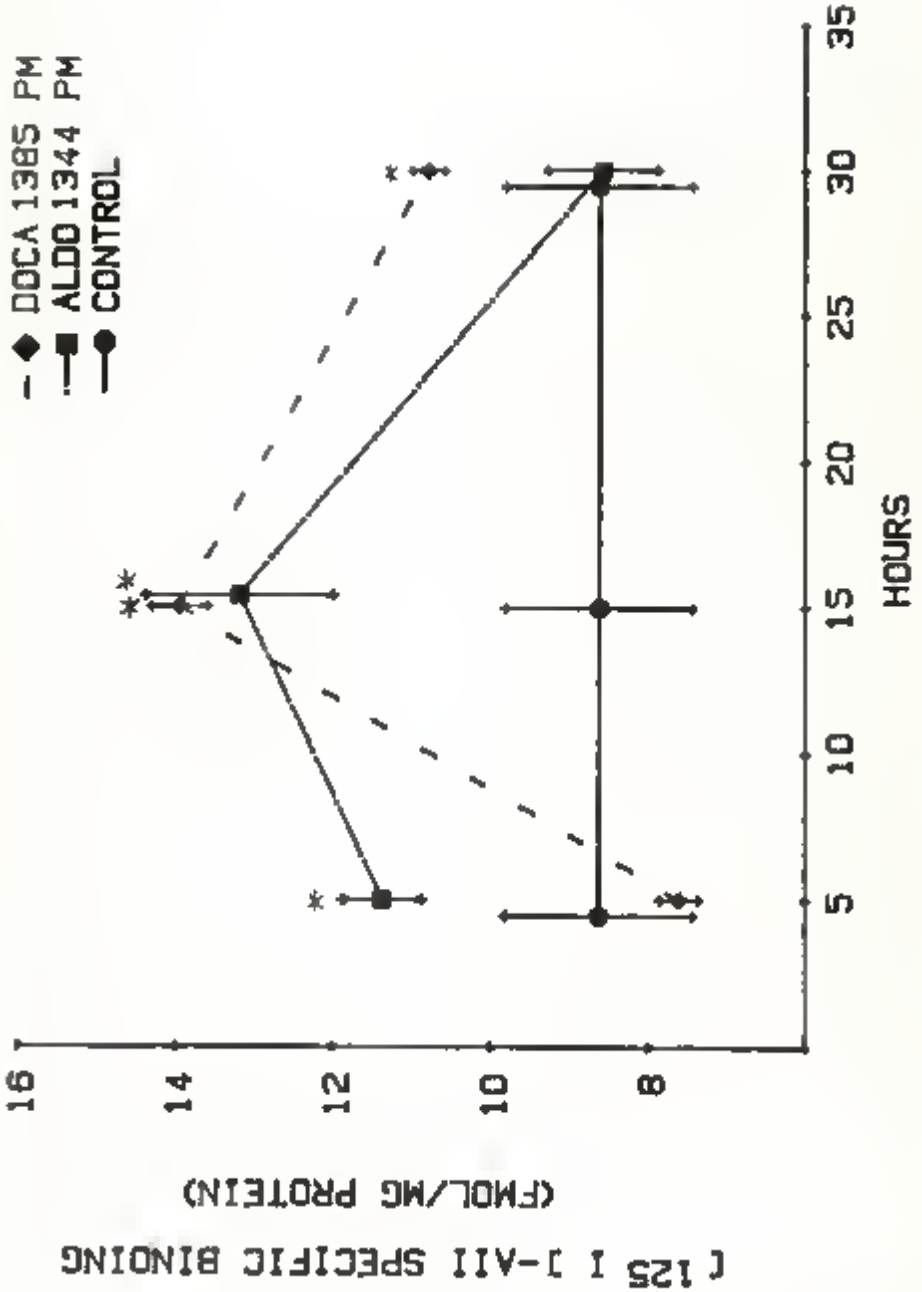

DOCA 1385 PM
ALDO 1344 PM
CONTROL
16
14
12
10
8
5
10
15
20
25
30
35
HOURS
(FMOL/MG PROTEIN)
[125 I]-AII SPECIFIC BINDING

Figure 28. Specific [^{125}I)-AII (0.25 nM) binding in brain neuronal cell cultures following treatment with different doses of either ALDO or DOCA (2.7-27,000 pM) for 20 hr. The dashed line represents control levels of specific AII binding. One SE is set off at each mean.
*Significantly different from control ($p<0.05$).
**Significantly different from control ($p<0.01$).

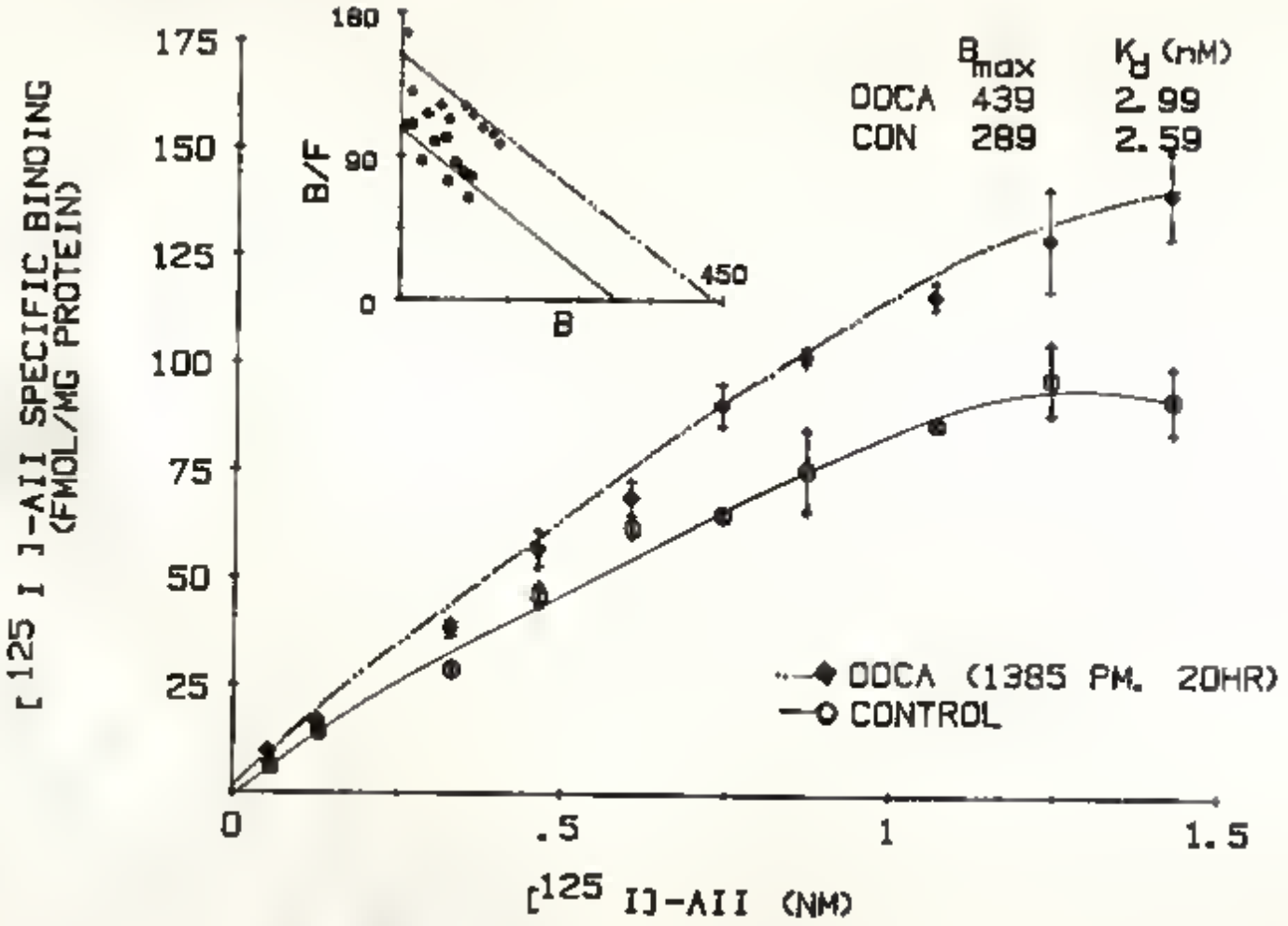
20 HR INCUBATION
DOCA
ALOO
CONTROL
[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
16
12
8
4
1
2
3
4
5
LOG [ALOO, OOCA] PM

Therefore, all subsequent studies were carried out at an incubation time of 20 hr.

At 20 hr, the specific binding of [^{125}I]-AII to neuronal cultures was augmented with increasing doses of either DOCA or ALDO (Fig. 28). A significant increase in binding was observed during treatment of neuronal cells with increasing concentrations of DOCA and ALDO up to 1300-1400 pM. Higher doses appeared to decrease binding back to control levels. The [^{125}I]-AII binding to cells treated with ALDO at concentrations of 2,700 - 27,000 pM was not significantly different from control. The binding of [^{125}I]-AII to DOCA-treated cells remained elevated above controls even with the highest dose of 28,000 pM.

To determine whether the increase in binding activity in DOCA-treated neuronal cultures was due to changes in the affinity and/or number of AII binding sites, specific binding was studied as a function of AII concentration. Figure 29 shows a representative saturation experiment. Triplicate control and DOCA-treated neuronal cultures were incubated with 0.05 to 1.5 nM [^{125}I]-AII at 24^{o}C for 60 min in either the presence or absence of unlabeled AII. Specific binding in both control and treated cultures saturated between 1.0-1.5 nM concentration of [^{125}I]-AII, although the binding in DOCA-treated cultures was significantly elevated above controls at concentrations above 0.25 nM. Scatchard analysis (Scatchard, 1949) of these data (upper graph, Fig. 29) gave straight lines for both cultures, which suggests homogeneous populations of AII-binding sites. The maximal number of binding sites in DOCA-treated neuronal cultures (B_{max} = 439 fmol/mg protein) was significantly elevated compared to control values (B_{max} = 288 fmol/mg protein), while the dissociation constants (K_d) were similar (DOCA: 2.99 nM, Controls: 2.59 nM).

Figure 29. Effects of increasing concentrations of [^{125}I]-AII on AII-specific binding in control and DOCA-treated (1385 pM, 20 hr) neuronal cultures prepared from brains of one-day-old rats. The lower panel represents saturation isotherms for [^{125}I]-AII specific binding in control and DOCA-treated cultures. One SE is set off at each mean. The upper panel illustrates a Scatchard analysis of the same data. B (fmol/mg protein) is the specific binding at each concentration of [^{125}I]-AII, and F is the free concentration (nM) of [^{125}I]-AII.


[125 I]-AII SPECIFIC BINDING (FMOL/MG PROTEIN)
175
150
125
100
75
50
25
0
.5
1
1.5
[125 I]-AII (NM)
B/F
180
90
0
450
B
Bmax
Kd (nM)
DOCA 439 2.99
CON 289 2.59
DOCA (1385 PM, 20HR)
CONTROL

Discussion

Chronic treatment with deoxycorticosterone acetate (DOCA) increased the dipsogenic responsiveness to both intraventricular and peripheral administration of AII by 45 and 80%, respectively. The pressor response induced by central administration of AII was also significantly greater in conscious DOCA-treated rats than controls by 48%. However, the pressor response elicited by s.c. administration of 10 ug AII/kg was not significantly greater than controls. When this dose was increased to 20 ug/kg, the response in DOCA-treated rats was again observed to be elevated 36% above controls.

The analysis of specific binding sites in brain tissue from the HTS revealed a significant increase in the maximal number of receptors in DOCA-treated rats, without a change in the dissociation rate (K_d). The levels of AII binding in the HTS of controls were similar to those reported by Sirett et al. (1977, 1982) and Mendelsohn et al. (1984).

Mann et al. (1981) reported a significant correlation between the affinity of several analogs of angiotensin for central angiotensin receptors and their effects on drinking, as well as their pressor responses. Decreases in AII receptor binding in estrogen-treated rats have also been related to the reduction in their dipsogenic response to AII (Fregly et al., 1985; Jonklaas and Buggy, 1985). The results presented here additionally suggest that an association may exist between the increased dipsogenic and pressor responsiveness to administration of AII and the increased number of binding sites for AII in the HTS of DOCA-treated rats. The absence of an enhanced pressor effect with the lower dose of AII administered s.c. may be related to the absence of a change in the affinity of AII for its receptors in the DOCA-treated rats. The augmented pressor response of the DOCA-treated rats which accompanied s.c. administration of the larger dose (20 ug/kg) may reflect

an increased number of AII receptors. However, it could also be argued that this may reflect peripheral receptor changes. It has been demonstrated that aldosterone can increase the number of AII receptors in mesenteric arteries in vivo and in vitro; however, responsiveness to administration of AII was not measured (Schiffrin et al., 1983a,b, 1984). In other studies, Monney et al. (1983) reported that AII induced an exaggerated contractile response in isolated perfused mesenteric arteries of DOCA-treated rats given either water or isotonic saline to drink. No differences in responsiveness to AII occurred in the saline-treated versus water-treated controls. Neither norepinephrine nor vasopressin was effective in eliciting a greater contractile response in the arteries of these DOCA-treated rats (Monney et al., 1983). In the present studies, the 10 ug dose of AII may not have been large enough to have a central effect; however, it has been suggested that acute increases in blood pressure greater than 35 mm Hg can open the blood brain barrier and thus allow passage of AII into the brain (Phillips, 1980). In either case, the increased dipsogenic and pressor responsiveness of DOCA-treated rats to central administration of AII was significantly greater than those following peripheral administration, although the centrally administered doses were one-tenth the peripheral dose. Therefore, the augmented responses to i.v.t. administration of AII suggest a relationship between central AII receptors and dipsogenic and pressor effects of centrally administered AII.

Studies by Douglas and Brown (1982) indicate that the number of AII receptors in the adrenal gland decreased during infusions of aldosterone. This change was compared to that observed during sodium loading (Aguilera and Catt, 1981; Douglas and Catt, 1976). A decrease in adrenal AII-receptor binding was also associated with a decrease in the AII-induced release of aldosterone from the adrenal cortex (Douglas and Brown, 1982; Douglas and Catt, 1976). In

mesenteric arteries, the bladder and the uterus, on the other hand, sodium loading tends to increase the binding of AII to its receptors, while sodium depletion has the opposite effect in each tissue (Aguilera et al., 1978; Catt et al., 1984; Gunther et al., 1980; Hauger et al., 1978).

The effect of alterations in sodium diets on AII receptors is thought to be mediated by the levels of AII in blood. However, the effect of peripheral levels of AII on central AII receptors is not clear, because it is proposed that AII cannot penetrate the blood brain barrier except at the fenestrated capillaries of the circumventricular organs (Phillips, 1980). Several studies have shown that the elevated plasma levels of AII induced by sodium restriction do not influence the binding of AII to its receptors in the brain (Cole et al., 1980; Husain et al., 1982; Sirett et al., 1982). However, studies by Mann et al. (1980b,c) reported a decrease in the number of central AII receptors accompanying sodium depletion. The animals also had a depressed pressor response to acute central administration of AII. Thus, the actual effect of the concentration of AII in peripheral circulation on angiotensin receptors in the brain is not known. In rats chronically treated with DOCA, the plasma levels of AII are severely depressed. If one assumes that the reduction of AII receptors during sodium depletion may be mediated by increases in plasma AII, the inverse relationship might be speculated with decreases in peripheral levels of AII. However, in estrogen-treated rats, the plasma levels of AII are also reduced, and the number of receptors in the HTS is decreased (Fregly et al., 1985; Jonklaas and Buggy, 1985). Therefore, these steroids may directly influence AII receptor binding.

To evaluate the relationship between mineralocorticoids and central AII receptors, without the potential influence of alterations in peripheral levels of AII, the specific binding of [^{125}I]-AII in brain neuronal cell cultures

was investigated. Previous studies have indicated that primary cultures prepared from fetal rat brains contained neurons with immunoreactive AII and specific AII binding sites on neuronal somites and neurites (Raizada et al., 1982; Stamler et al., 1981). In the present study, the binding of [^{125}I]-AII to either ALDO- or DOCA-treated neuronal brain cell cultures resulted in a time- and dose-dependent increase above that of controls. Treatment with ALDO stimulated a significant increase in binding by 5 hr, while an increase was not observed with DOCA until 15 hr. The enhanced levels of [^{125}I]-AII binding in cultures treated with either DOCA or ALDO at 20 hr were similar except at the higher doses. Cultures treated with DOCA continued to show increases in AII binding, while no significant increases were elicited with the higher doses of ALDO. Scatchard analysis revealed that the increase observed in DOCA-treated cultures was due to a two-fold increase in the maximal number of AII binding sites (B_{max}), while the affinity (K_d) of AII for its binding site was similar in DOCA-treated cells and controls. Thus, an increase in the number of AII binding sites is observed following treatments with mineralocorticoids in both brain membrane and neuronal cell preparations in the absence of a change in affinity.

Other studies have demonstrated the ability of cells to incorporate [^{3}H]-valine and [^{3}H]-isoleucine into immunoprecipitable [^{3}H]-AII and suggest that neuronal cells in culture may be able to synthesize AII (Raizada, 1983). If the increased binding of AII to cultured neurons induced by DOCA resulted from an increase in either the synthesis or release of AII, it might be speculated that saralasin, an AII receptor antagonist, could block the effect of DOCA on AII receptors. Preliminary experiments indicate failure of saralasin to do so. The absence of an effect by pretreatment with saralasin suggests that DOCA may directly affect either the binding of AII at its receptor or the mechanisms activated by receptor occupancy. The number of AII

binding sites was increased in neuronal cell cultures prepared from the brains of one-day-old spontaneously hypertensive rats (SHR) compared to the cultures prepared from the brains of WKY controls, while the concentration of AII in the cultures was reduced (Raizada et al., 1984a). The low concentration of AII is thought to influence the increase in the binding of AII to its receptors. Thus, the possibility also exists that DOCA may decrease AII levels in culture and thus increase binding. The mechanism by which DOCA up-regulates AII receptors cannot be ascertained from the data presented here, but is presently under investigation in this laboratory. However, the ability of DOCA to initiate a response in cell culture, which is similar to that seen in vivo, suggests that the effect of DOCA on AII receptors in the brain is independent of its effect on circulating levels of AII.

CHAPTER VII
EFFECTS OF INCREASED CIRCULATING ANGIOTENSIN II ON FLUID BALANCE AND CENTRAL ANGIOTENSIN II RECEPTORS IN RATS

Introduction

The role of the renin-angiotensin system (RAS) in the regulation of blood pressure and fluid and electrolyte homeostasis is dependent upon the ability of angiotensin II (AII) to interact with receptors in peripheral tissues and the central nervous system (Fitzsimons, 1980; Ramsay, 1982; Reid, et al., 1978). In the periphery, alterations in the levels of circulating AII have been shown to affect the binding of AII to its receptors in the adrenal and vascular smooth muscle, and thus the responsiveness of these tissues to release aldosterone and vasoconstrict, respectively (Aguilera et al., 1978; Gunther et al., 1978; Douglas and Brown, 1982; Catt et al., 1984). However, the influence of the peripheral RAS on the state of AII receptors in the brain remains undefined.

The hypothalamus, thalamus, and septum contain high concentrations of AII receptors which are proposed to mediate the dipsogenic and pressor responses elicited by AII (Sirett et al., 1977, 1979; Mann et al., 1980a, 1981). However, many of these receptors are distinctly found on the circumventricular organs (Sirett et al., 1977; Mendelsohn et al., 1984; Phillips, 1978). These areas, which include the subfornical organ (SFO), the organum vasculosum of the lamina terminalis (OVLT) and the median eminance (ME), lack the the tight junctions which preclude entry of polar and high molecular weight substances into the central nervous system (CNS) and are accessible to circulating AII (Weindl, et al., 1973; Phillips, 1978; Van Houten et al., 1980). Much controversy exists as to whether AII can cross the blood brain barrier because of its polar

nature. Thus, the functional significance and regulation of AII receptors within the BBB, which are also indicated to mediate pressor and dipsogenic responses elicited by AII (Brody and Johnson, 1980; Phillips, 1980), remain enigmatic.

The effect of alterations of circulating AII induced by changes in sodium concentrations in the diet on AII receptors in the central nervous system has been investigated; however, the results are controversial. Sodium loading, which reduces circulating levels of AII does not appear to have any influence on central AII receptors (Mann et al., 1980a; Cole et al., 1980). Conversely, when endogenous levels of AII have been elevated by a sodium deficient diet, either an increase (Thomas and Sernia, 1985), a decrease (Mann et al., 1980b) or no change may occur in the number of AII receptors in the brain (Cole et al., 1980; Husain et al., 1982; Sirett et al., 1982). As demonstrated in the previous chapter, AII receptors in the hypothalamus, thalamus and septum (HTS) are increased by treatment with a mineralocorticoid that reduces plasma renin activity. AII also has been infused directly into the brain to determine whether exogenous AII can alter either central or peripheral AII receptor binding. No change was observed in either the brain or adrenal (Singh et al., 1984). Thus, the question remains as to whether AII can regulate its own receptors in the brain.

Several studies have shown that i.v.t. infusions of AII are accompanied by dramatic rises in blood pressure, water intake and urine output. The alterations in fluid balance have been shown to either subside during the infusion period (Gronan and York, 1979; DiNicolantonio et al., 1982; Singh et al., 1984) or remain elevated through the duration of the study from 5 to 20 days (Phillips et al., 1979; Sterling, et al. 1980, Fink et al., 1982; Rettig et al., 1984). In dogs, chronic i.v. infusions are also accompanied by sustained increases in blood pressure, water intake and urine output. However,

in rats, Fink and Bruner (1985) have demonstrated that the changes in fluid balance are not elicited through chronic i.v. infusions of AII with doses up to 60 ng/min, i.v.

Thus, the effect of alterations in circulating AII upon receptors in the CNS and fluid balance is still not clear. The purpose of these studies was to determine whether s.c. infusions of AII could influence the binding of AII to its receptors in the brain, and whether a relationship between the state of central AII receptors and the changes in both daily fluid balance and blood pressure could be established. Since mineralocorticoids are also elevated during chronic AII infusions, additional studies were conducted to determine the extent of mineralocorticoid involvement in the regulation of AII receptor binding capacity.

Methods

Experiment 1. Effect of Chronic Angiotensin II Infusions on Sodium Appetite and the Dipsogenic Responsiveness to Acute Administration of Angiotensin II.

Fourty-four female rats and 14 male rats weighing 250-350 g were used. Three separate studies were conducted. In each of these studies, half of the rats was anesthetized with ether and Alzet osmotic mini-pumps were implanted subcutaneously between the shoulder blades. AII was dissolved in 0.9% sterile saline and and placed in the mini-pump to deliver either 40 or 125 ng/kg/min, s.c. (2.4 and 7.5 ug/kg/hr). The other half was anesthetized and received a s.c. incision. Initial experiments showed no difference between rats implanted with saline-filled mini-pumps and sham-operated rats.

In the first study, 16 female rats received s.c. infusions of AII at 40 ng/kg/day for 2 weeks. On day 2, the drinking response to s.c. administration of either AII (200 ug/kg) or isoproterenol (25 ug/kg) was tested in AII-infused rats and their controls. Water intakes were measured gravimetrically at 0.5, 1 and 2 hr after administration of AII. To test the effect of AII infusions on sodium appetite, the groups were randomly divided in half on day 5, so that 8

AII-infused and 8 controls were given a choice between water and 0.15 M NaCl. The other 2 groups of rats were given a choice between water and 0.25 M NaCl. Water and NaCl intakes were measured for two consecutive 24 hour periods. On day 7, half of the rats was implanted with intracerebroventricular cannulae and all were given new pumps. On days 9 and 12, the drinking responses to s.c. and i.v.t administration of AII, respectively, were examined.

In the next study, 12 female rats were centrally cannulated a week before the infusion was started. Six of these rats were infused with AII at a rate of 125 ng/kg/min for 6 days. On the second day of the infusion period, AII-infused rats and their controls were tested for their dipsogenic responsiveness to s.c. administration of AII. On the fifth day, the drinking response to i.v.t. administration of AII was tested. This study was repeated in male rats to assess whether the ovarian cycle influenced the responses. No differences were noticed between males and females, so the data were combined.

Experiment 2. Effect of Angiotensin II-infusions on Angiotensin II Receptor Binding in the MTS

Eighteen male rats were used in the first portion of this study and housed in individual metabolic cages one week prior to the infusion period. Half of the rats was implanted with osmotic mini-pumps which delivered 125 ng AII/kg/min, s.c. Food and water intakes and urine outputs were measured daily. Systolic blood pressure was measured prior to the AII infusion and on days 2 and 4 during the five-day infusion period, by the tail-cuff method as described previously (Fregly, 1963).

After 5 days, all rats were killed by guillotine decapitation. Blood was collected in EDTA and centrifuged at 4°C. Plasma was seperated and stored at 4°C until determination of aldosterone and plasma renin activity (PRA) by radioimmunoassay. The brain was removed and a block of tissue containing

the hypothelamus, thalamus, and septum was diasected free for AII-receptor binding as described in the general methods.

The same protocol was repeated as above in 24 female rats except that the effects of two infusion rates, 40 ng AII/kg/min and 125 ng AII/kg/min, were analyzed.

Experiment 3. Effect of Adrenalectomy on Angiotensin II Receptor Binding Capacity in Control and Angiotensin II-Infused Rats

Sixteen male rats were used for this study. After a three day control period, the rats were anesthetized with ether, and either bilaterally adrenalectomized, implanted with an Alzet osmotic mini-pump, or bilaterally adrenalectomized and implanted with a mini-pump. Thus, 4 equal groups were established: (1) sham controls; (2) rats infused with 125 ng AII/kg/min,s.c.; (3) adrenalectomized; (4) adrenalectomized + an infusion of 125 ng AII/kg/min, s.c. During the six days of treatment, all rats were provided with 0.9% NaCl solution as their sole drinking fluid. Urine volume and food and water intakes were measured daily. At the end of this period, all rats were killed and the BFS was removed for analysis of AII receptor binding capacity. Blood was collected in EDTA for determination of aldosterone and PRA by radioimmunoassay.

Results are expressed as means ± one standard error. Statistical significance was calculated by one-way analysis of variance, and Student's paired and unpaired t-tests. Regression lines were calculated by the method of least squares (Zar, 1974).

Results

Experiment 1.

At an infusion rate of 40 ng AII/kg/min, the drinking response to either subcutaneous or central administration of AII at 0.5, 1, and 2 hr was not different from controls. Table 4 summarizes the effects of chronic infusions

TABLE 4

I. EFFECT OF ACUTE SUBCUTANEOUS (s.c.) AND INTRAVENTRICULAR (i.v.t.) ADMINISTRATION OF ANGIOTENSIN II (AII) AND ISOPROTERENOL ON 2 HR WATER INTAKES IN CONTROL RATS AND RATS RECEIVING CHRONIC INFUSIONS OF AII AT EITHER 40 OR 125 NG/KG/MIN.

TREATMENT	INTAKE (ml/kg at 2 hr)		OUTPUT (ml/kg at 2 hr)	
	CONTROL	AII	CONTROL	AII
A. 40 ng AII/kg/min (n=32)				
200 ug AII/kg, s.c.	14.1 ± 2.3+	18.7 ± 2.2	11.8 ± 2.4	19.2 ± 2.6*
25 ug ISO/kg, s.c. (2nd week)	10.4 ± 2.5	14.8 ± 2.3	7.9 ± 2.5	8.4 ± 2.5
200 ug AII/kg, s.c.	21.0 ± 3.1	17.6 ± 3.4	11.7 ± 1.2	13.3 ± 1.9
10 ng AII, i.v.t.	18.9 ± 5.4	25.8 ± 6.8	8.3 ± 3.5	9.6 ± 3.0
B. 125 ng AII/kg/min (n=14)				
100 ug AII/kg, s.c.	13.5 ± 3.5	16.8 ± 3.5	10.0 ± 2.2	13.4 ± 2.9
10 ng AII, i.v.t.	24.9 ± 7.1	17.4 ± 4.6	14.1 ± 4.6	13.0 ± 4.6

II. EFFECT OF CHRONIC AII INFUSIONS (40 NG/KG/MIN) ON SODIUM APPETITE. RATS WERE GIVEN A CHOICE OF WATER AND EITHER 0.15 M OR 0.25 M NACL FOR 48 HR. VALUES REPRESENT THE MEAN (ML/KG B.W.) OF TWO-24 HR INTAKES (n=8/GROUP).

Group	Intakes: Water	0.15 M NaCl	0.25 M NaCl
Control	147.4 ± 20.1+	67.9 ± 13.1	
AII	118.3 ± 12.1	107.4 ± 16.1 *	
Control	169.0 ± 10.5		68.9 ± 10.8
AII	149.2 ± 10.6		43.0 ± 6.1

+ One standard error of mean.
* Significantly different from control ($p<0.05$)

of AII in rats on their acute dipsogenic responsiveness to either AII or isoproterenol. The dipsogenic response to administration of isoproterenol was also unaffected by chronic treatment with AII. In the first test, with acute administration of AII, urine output was enhanced in the AII-infused group; but with subsequent tests, this difference disappeared. In the second week of treatment, the drinking response to i.v.t. AII tended to increase, but this was not significant. Therefore, the AII infusion was increased to 125 ng/kg/min for the next study. However, as observed in Table 4, the drinking response to both s.c. and i.v.t. administered AII was again unaffected by chronic treatment with AII at this higher dose.

The effect of chronic infusions of AII (40 ng/kg/min) on sodium appetite was also analyzed. As observed in the second half of Table 4, the intake of NaCl by AII-infused rats given a choice between tap water and 0.15M NaCl was significantly greater than their respective controls. However, in rats given a choice between water and 0.25M NaCl, the animals treated with AII appeared to drink less NaCl than the controls, although this difference was not significant.

Experiment 2.

Chronic administration of AII at either 40 ng/kg/min or 125 ng/kg/min significantly increased daily water intake, urine output, and blood pressure. Table 5 summarizes the mean change in these parameters for each group studied. Although not shown, at the infusion rate of 40 ng/kg/min, water intake gradually increased over the treatment period, so that by day 4, significant differences were observed between groups, but this difference was not reflected in the mean daily intake compared to controls. Urine output, on the other hand, increased by day 2 and remained elevated so that the mean urine output accompanying AII infusions of 40 ng/kg/min was significantly different from controls.

TABLE 5

EFFECT OF CHRONIC S.C. INFUSIONS OF ANGIOTENSIN II ON MEAN WATER INTAKE, URINE OUTPUT, AND BLOOD PRESSURE. EACH VALUE REPRESENTS THE AVERAGE OF THE 5 DAYS OF TREATMENT FOR EACH GROUP.

TREATMENT	WEIGHT (kg)	WATER INTAKE (ml/kg)	URINE OUTPUT (ml/kg)	BLOOD PRESSURE (mm Hg)
A. Females				
40 ng AII/kg/min	0.229	139.9 ± 9.8+	70.7 ± 6.8 *	129 ± 6*
controls	0.223	122.7 ± 8.2	51.8 ± 4.8	110 ± 5
difference		17.2 (12%)	18.9 (27%)	18.7 (15%)
B. Females				
125 ng AII/kg/min	0.240	163.7 ± 9.6*	80.3 ± 7.1**	128 ± 5*
controls	0.234	127.2 ± 6.3	45.0 ± 6.0	116 ± 4
difference		36.5 (22%)	35.3 (44%)	12 (10%)
C. Males				
125 ng AII/kg/min	0.341	143.2 ± 10.2*	70.9 ± 6.9**	143 ± 5**
controls	0.352	114.6 ± 8.1	42.8 ± 3.8	113 ± 3
difference		28.6 (20%)	28.1 (40%)	29.4 (21%)

\+ One standard error of mean
* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)
(% change from control)

Figure 30 illustrates the effect of AII infusions at 125 ng/kg/min on daily fluid exchanges in male rats. Although water intake (Panel A) and urine output (Panel B) are elevated during treatment, both groups remain in water balance (Panel C). The effect of this dose of AII on water intake and urine output was almost identical when responses of males and females were compared. Hence, the results of the study using female rats are not presented. Blood pressure also increased significantly in both sexes (Table 5).

The binding of AII to membranes prepared from the HTS of control and AII-infused (125 ng/kg/min) rats was performed at both 0.25 and 1.0 nM [^{125}I]-AII in the incubation medium. In AII-infused rats, the specific binding of [^{125}I]-AII to brain membranes was significantly increased compared to controls (Fig. 31 and 32). For male rats, this increase above controls was 50% at 0.25 nM and 27% at 1.0 nM [^{125}I]-AII in the incubation medium. In female rats, this increase was 31% and 33% respectively.

The relationship between the increases in water intake, urine output, and blood pressure, and the increase in AII receptor binding in the HTS of male rats treated with 125 ng AII/kg/min, s.c. was determined by the least squares method (Zar, 1974). To avoid any controversy with respect to any potential effect of ovarian hormones on fluid balance, this analysis of the data was performed only for the males. As is observed in Fig. 33, water intake and the specific binding of [^{125}I]-AII to receptors in the HTS of AII-infused rats were highly correlated ($r = 0.94$, $p<0.001$). Likewise, the correlation between urine output and specific AII binding was also significant ($r = 0.69$, $p<0.05$) (Fig. 34). However, the increase in blood pressure was not related to the increase in AII receptor binding in the HTS accompanying infusions of AII (Fig. 35). In control rats, similar relationships were observed: water intake and urine output were significantly correlated with AII receptor binding in the HTS

Figure 30. Mean water intake (A) and urine output (B) of controls and rats infused with 125 ng AII/kg/min, s.c. Panel C represents the mean water balance (exchange) during the treatment period. One SE is set off at each mean.

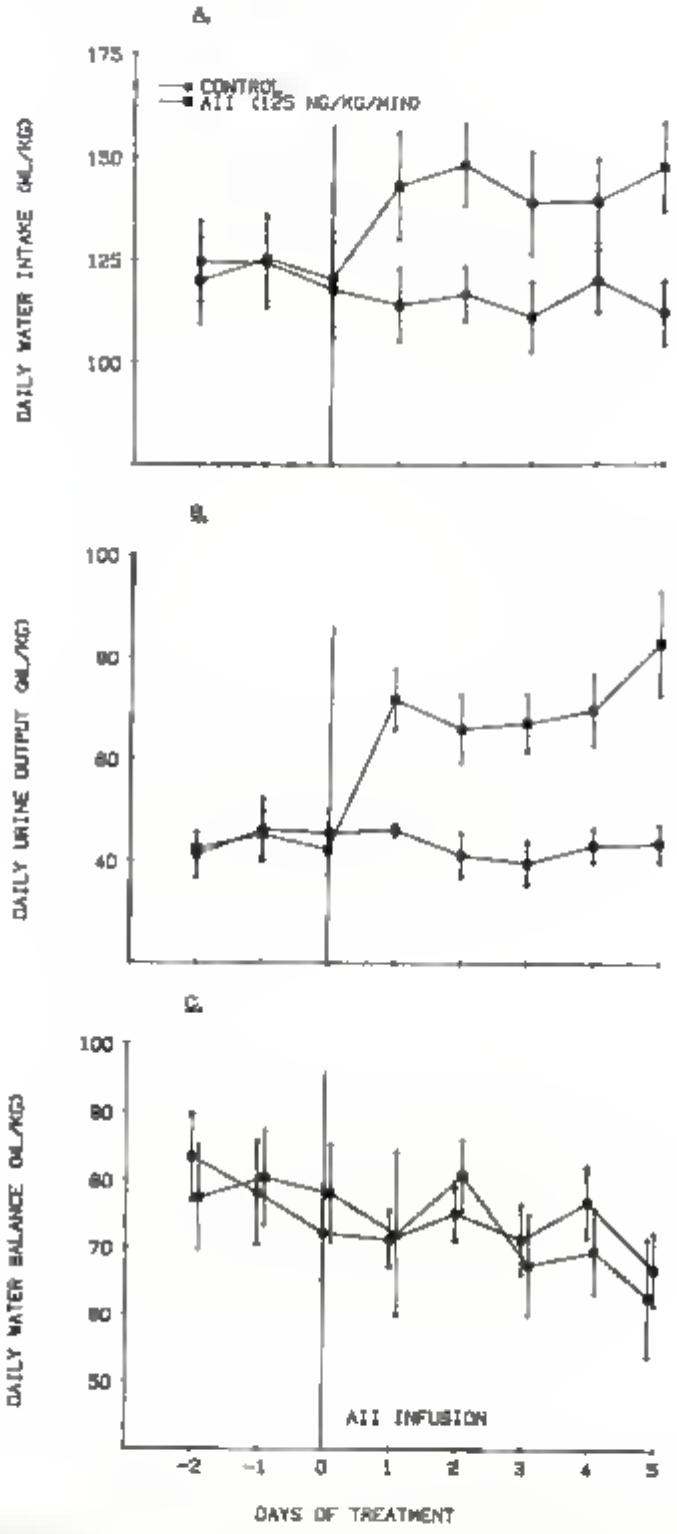

A.
175
150
125
100
CONTROL
AII (125 NG/KG/MIN)
DAILY WATER INTAKE (ML/KG)
B.
100
80
60
40
DAILY URINE OUTPUT (ML/KG)
C.
100
90
80
70
60
50
DAILY WATER BALANCE (ML/KG)
AII INFUSION
-2
-1
0
1
2
3
4
5
DAYS OF TREATMENT

Figure 30. Mean water intake (A) and urine output (B) of controls and rats infused with 125 ng AII/kg/min, s.c. Panel C represents the mean water balance (exchange) during the treatment period. One SE is set off at each mean.

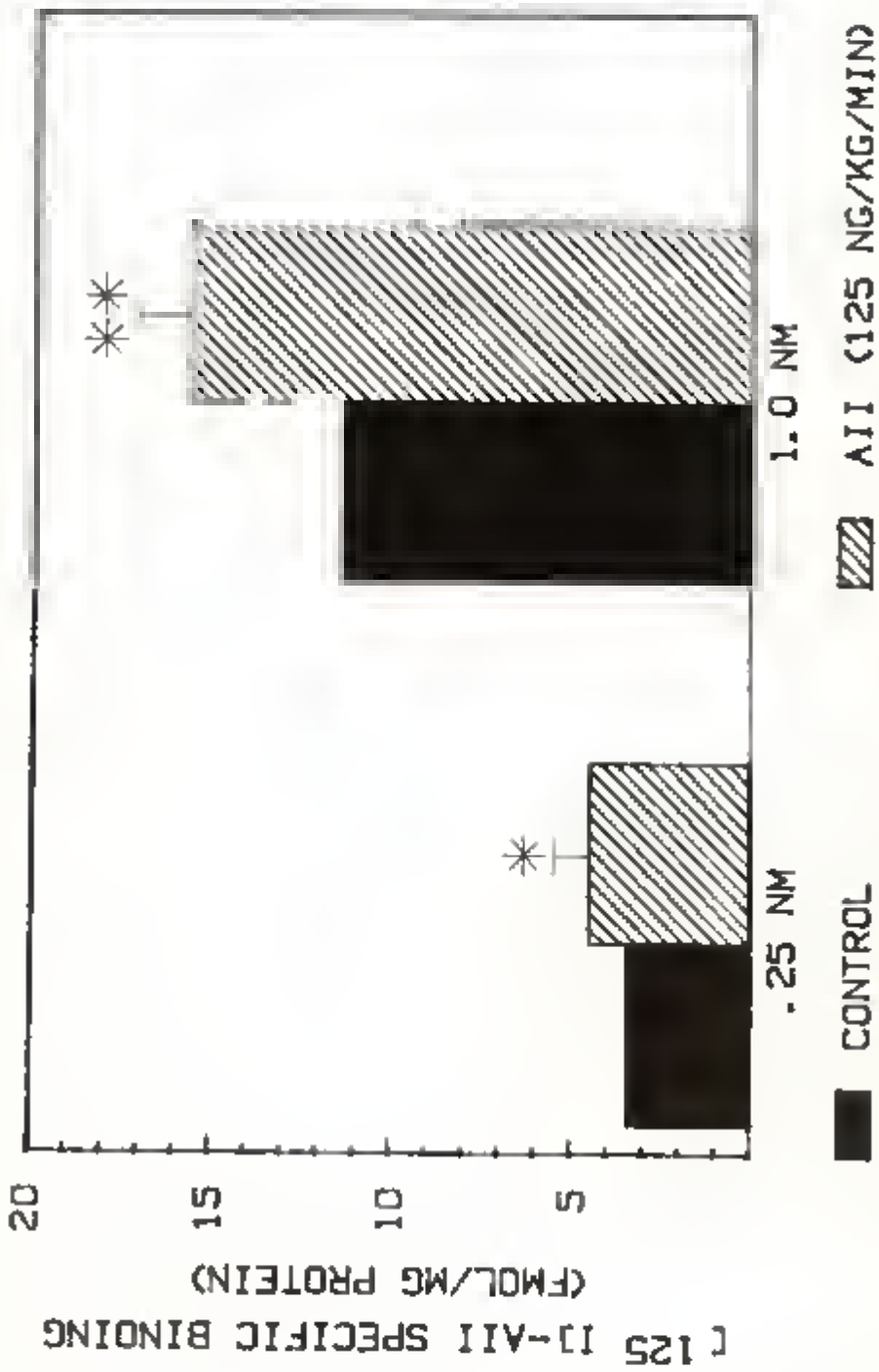

[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
20
15
10
5
.25 NM
1.0 NM
CONTROL
AII (125 NG/KG/MIN)

Figure 32. Effect of chronic infusions of AII (125 ng/kg/min, s.c.) to female rats for 5 days on the specific AII binding at 0.25 and 1.0 nM concentrations of [I^{125}]-AII in membranes prepared from the MTS of control and AII-treated rats. The y-axis represents AII specific binding, and each bar is the average of individual binding results (n = 4). One SE is set off at each mean. *Significantly different from control ($p<0.05$). **Significantly different from control ($p<0.01$).

[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
25
20
15
10
5
.25 NM
1.0 NM
CONTROL
AII (125 NG/KG/MIN)

Figure 33. Relationship between [^{125}I]-AII receptor binding (1 nM) in the HTS of rats treated with 125 ng AII/kg/min, s.c. and the mean daily water intake during the 5-day infusion period.

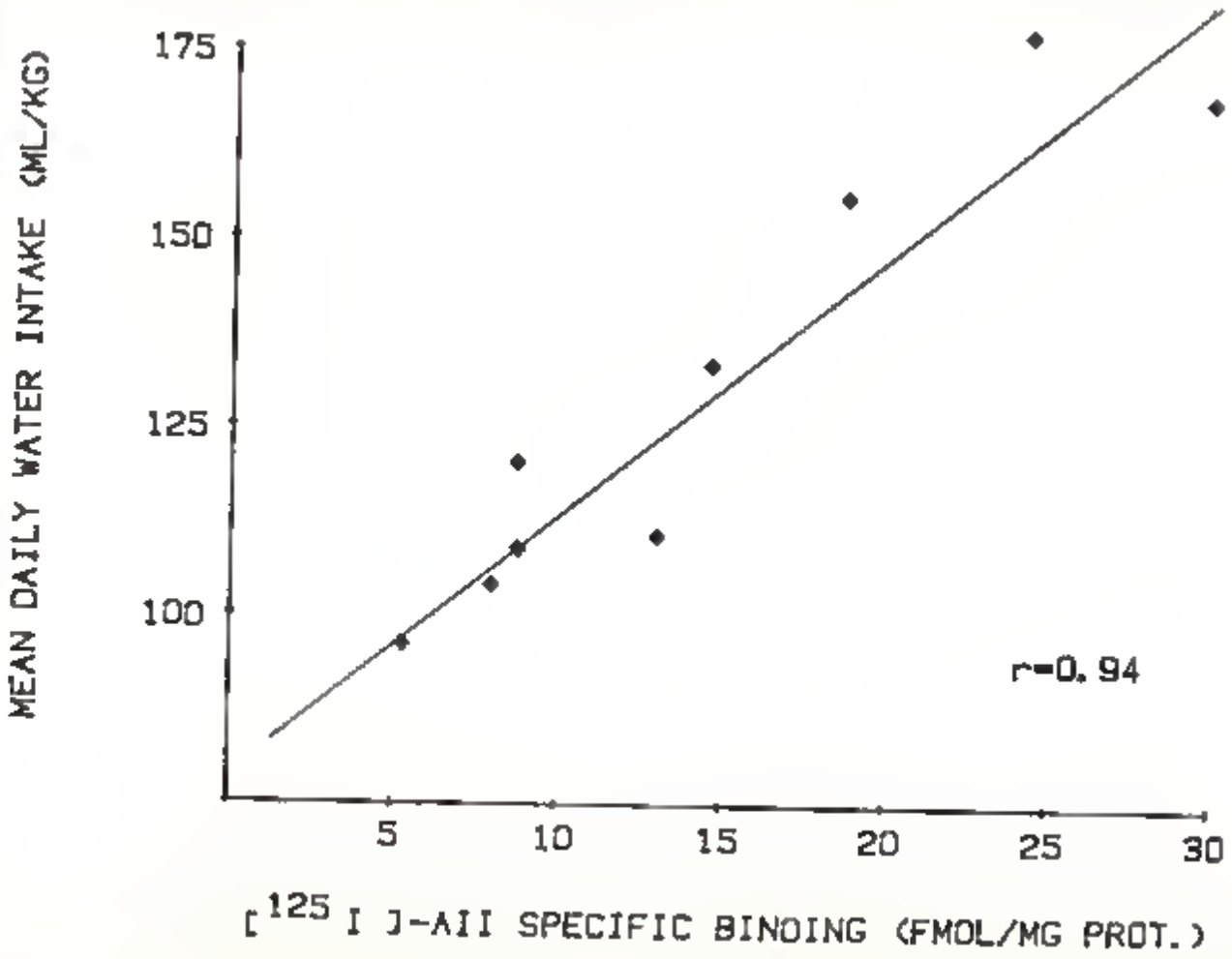

MEAN DAILY WATER INTAKE (ML/KG)
175
150
125
100
r=0.94
5
10
15
20
25
30
[125 I]-AII SPECIFIC BINDING (FMOL/MG PROT.)

Figure 34. Relationship between [^{125}I]-AII receptor binding (1 nM) in the HTS of rats treated with 125 ng AII/kg/min, s.c. and the mean daily urine output during the 5-day infusion period.

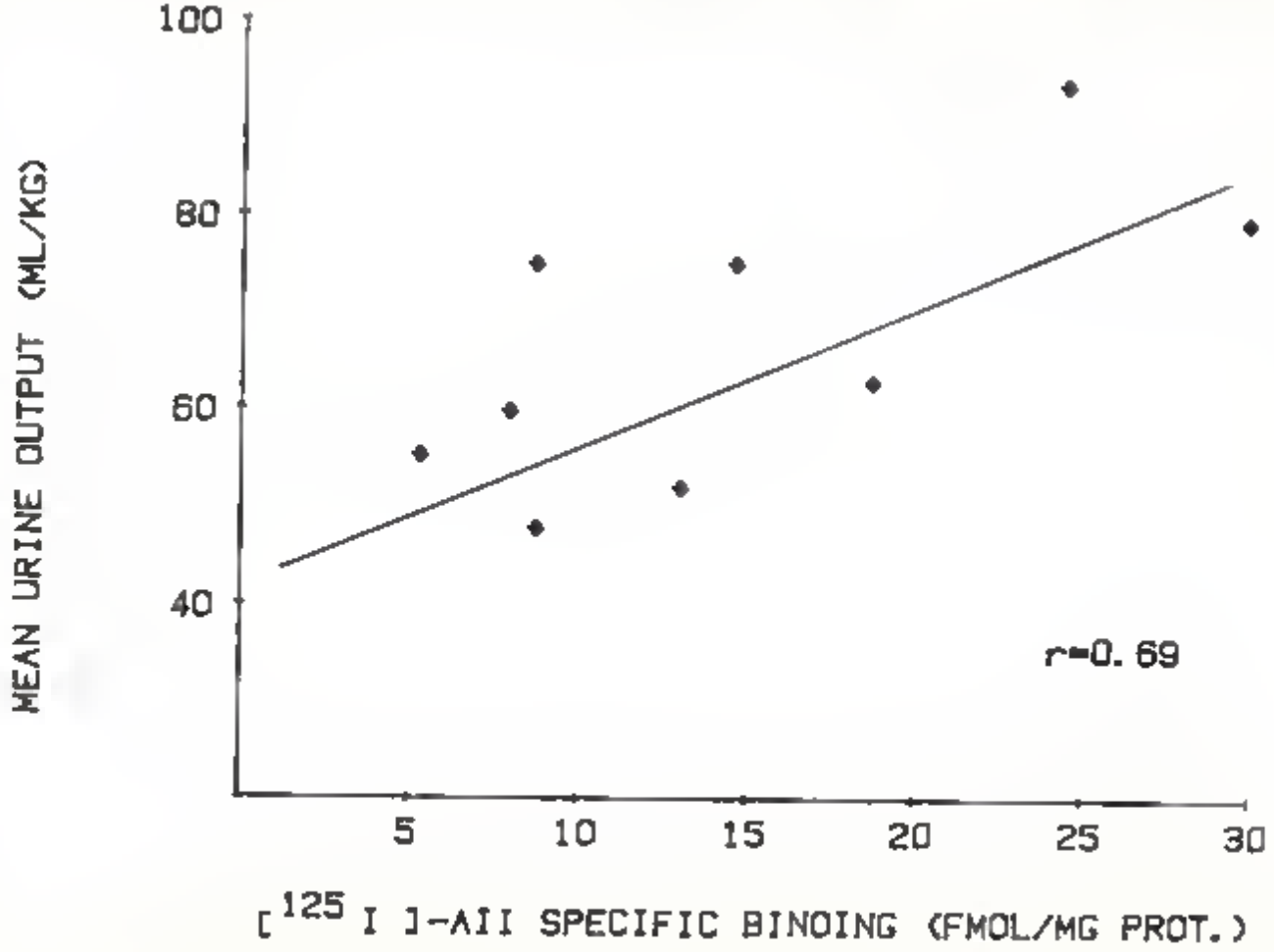
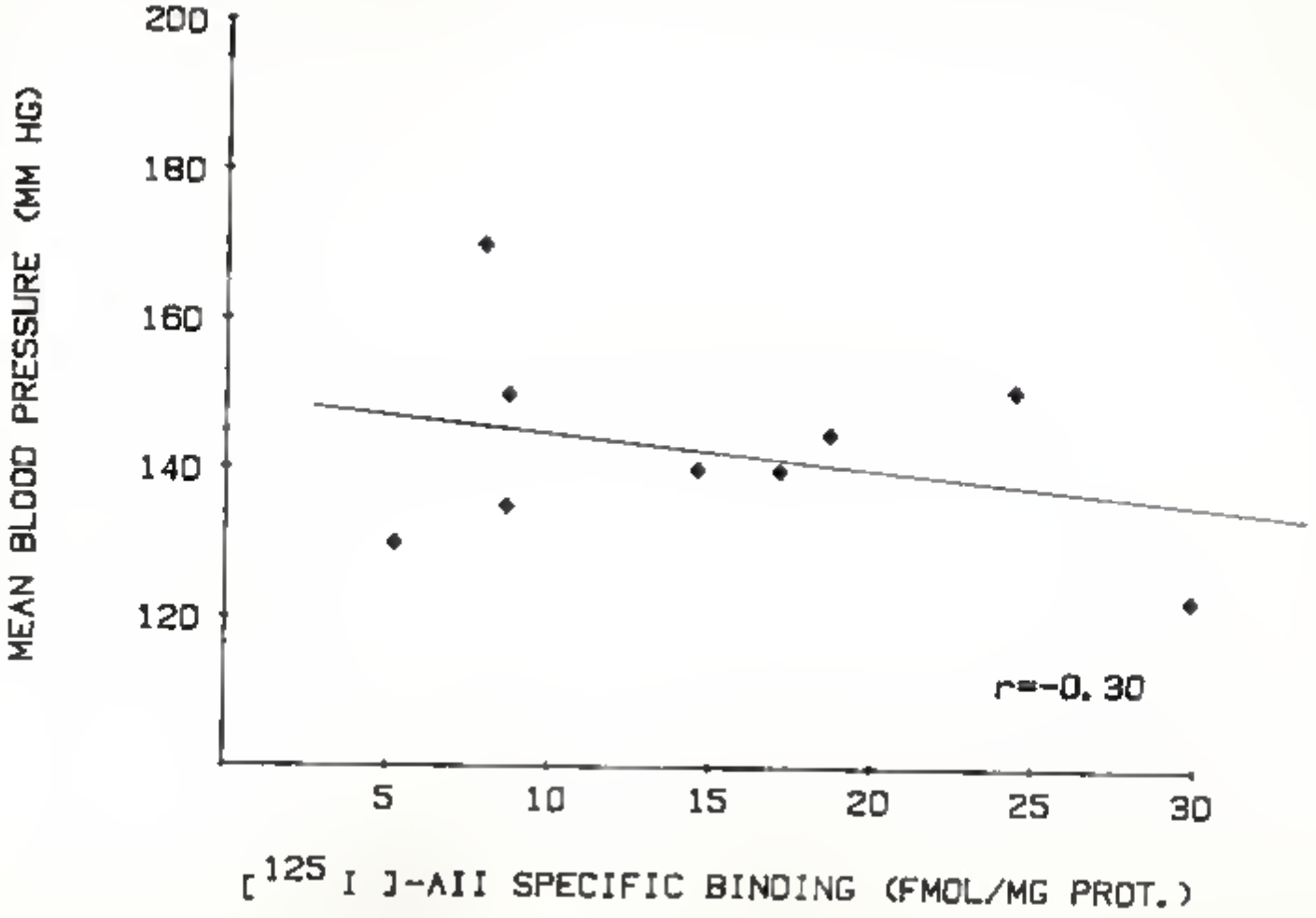

100
80
60
40
MEAN URINE OUTPUT (ML/KG)
r=0.69
5
10
15
20
25
30
[125 I]-AII SPECIFIC BINDING (FMOL/MG PROT.)

Figure 35. Relationship between [^{125}I]-AII receptor binding (1 nM) in the HTS of rats treated with 125 ng AII/kg/min, s.c. and the mean blood pressure during the 5-day infusion period.


MEAN BLOOD PRESSURE (MM HG)
200
180
160
140
120
r=-0.30
5
10
15
20
25
30
[125 I]-AII SPECIFIC BINDING (FMOL/MG PROT.)

(r = 0.79 and 0.65, respectively, p<0,05), while the mean blood pressure of controls was not related (r = 0.4).

To determine whether the increase in binding was due to an increase in either the number or affinity of AII binding sites, the specific binding of [^{125}I]-AII to the HTS from AII-treated (40 ng/kg/min) and control rats was studied as a function of AII concentration (Fig. 36). Each point represents AII specific binding from the pooled tissue (HTS) of 4 rats. After a 45 min incubation period, the specific binding reached a plateau between 0.6 and 1.0 nM in both control and AII-infused rats. Specific binding to receptors in the HTS of AII-treated rats was increased at most concentrations of AII compared to controls. Scatchard analysis (Scatchard, 1949) of these data indicated that the increase in binding of AII to receptors in the brain of the AII-infused rats was due to a increase in the number of binding sites (B_{max} = 12.13 vs. 8.79) and no change in affinity. The increase in the number of AII receptors in the AII-infused group was statistically significant as determined by comparison of two elevations (p<0.05) (Zar, 1974).

Experiment 3.

The fluid intake (Panel A) and urine output (Panel B) of the controls, adrenalectomized rats, and rats infused with 125 ng AII/kg/min were increased throughout the treatment period (saline was their sole drinking fluid) (Fig. 37). A significant treatment X urine output interaction was observed between groups (p<0.01) as well as water intake and urine output X days interaction (p<0.05). Panel C represents the effect of the treatment on water balance. A significant interaction (p<0.01) of water balance X treatment was observed.

A summary of the effect of AII infusions (125 ng/kg/min) on plasma renin activity (PRA) and aldosterone concentration is presented in Table 6. Infusions of AII induced a significant increase in aldosterone release and a

Figure 36. The effect of increasing concentrations of [^{125}I]-AII on the specific binding of [^{125}I]-AII to membranes prepared from the hypothalamus, thalamus and septum (HTS) of control rats and rats receiving AII infusions (40 ng/kg min, s.c.). The lower panel represents saturation isotherms for [^{125}I]-AII specific binding in membranes prepared from AII-treated and control rats (n = 4). One SE is set off at each mean. The upper panel illustrates a Scatchard analysis of the same data. B (fmol/mg protein) is the specific binding at each concentration of [^{125}I]-AII, and F is the free concentration (nM) of [^{125}I]-AII.

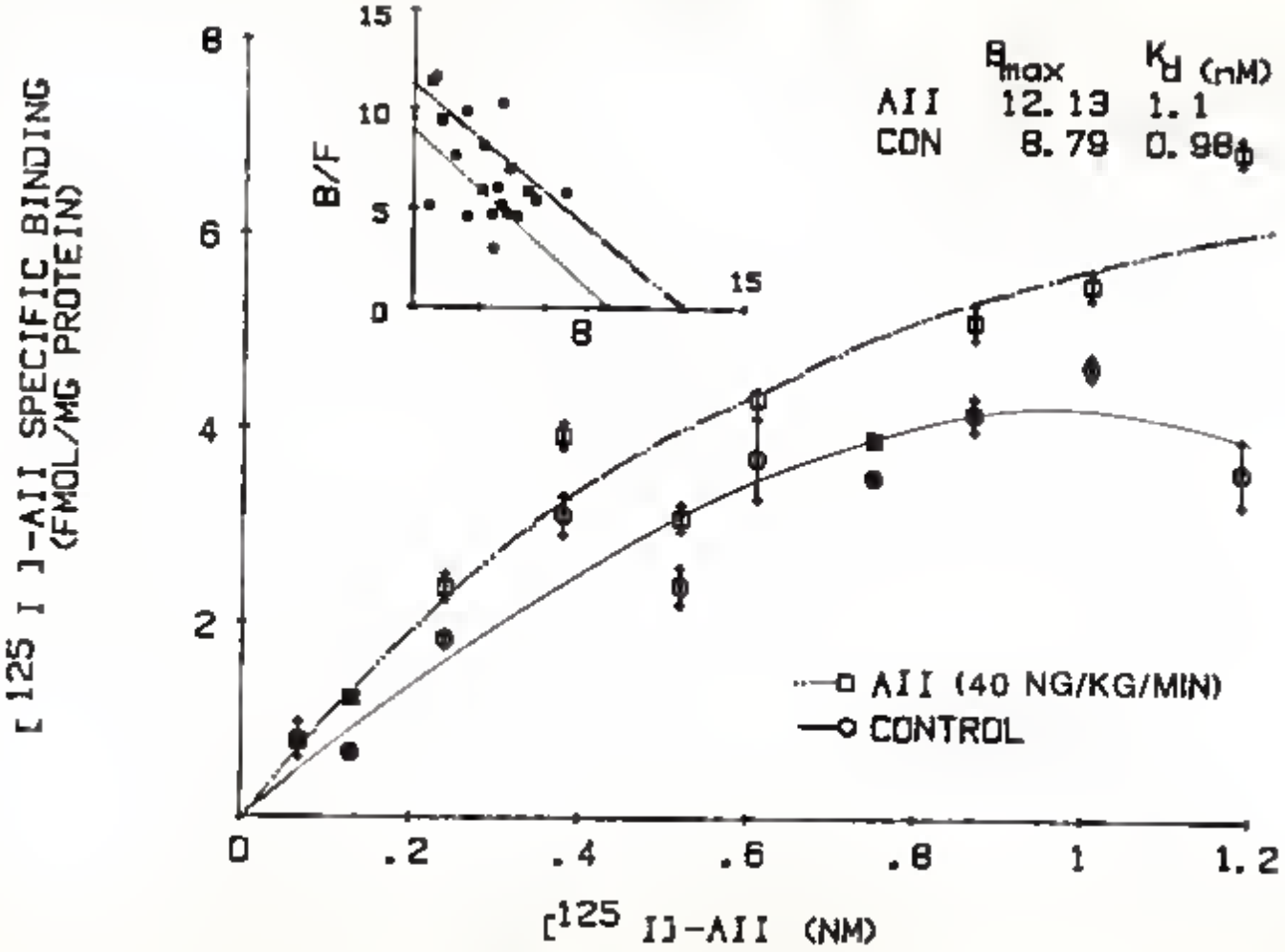

[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
8
6
4
2
0
.2
.4
.6
.8
1
1.2
[125 I]-AII (NM)
B/F
15
10
5
0
15
8
Bmax
Kd (nM)
AII 12.13 1.1
CON 8.79 0.98
AII (40 NG/KG/MIN)
CONTROL

Figure 37. Mean water intake (A) and urine output (B) of control and adrenalectomized rats infused with 125 ng AII/kg/min, s.c. Panel C represents the mean water balance (exchange) for the treatment period of 6 days. One SE is set off at each mean.

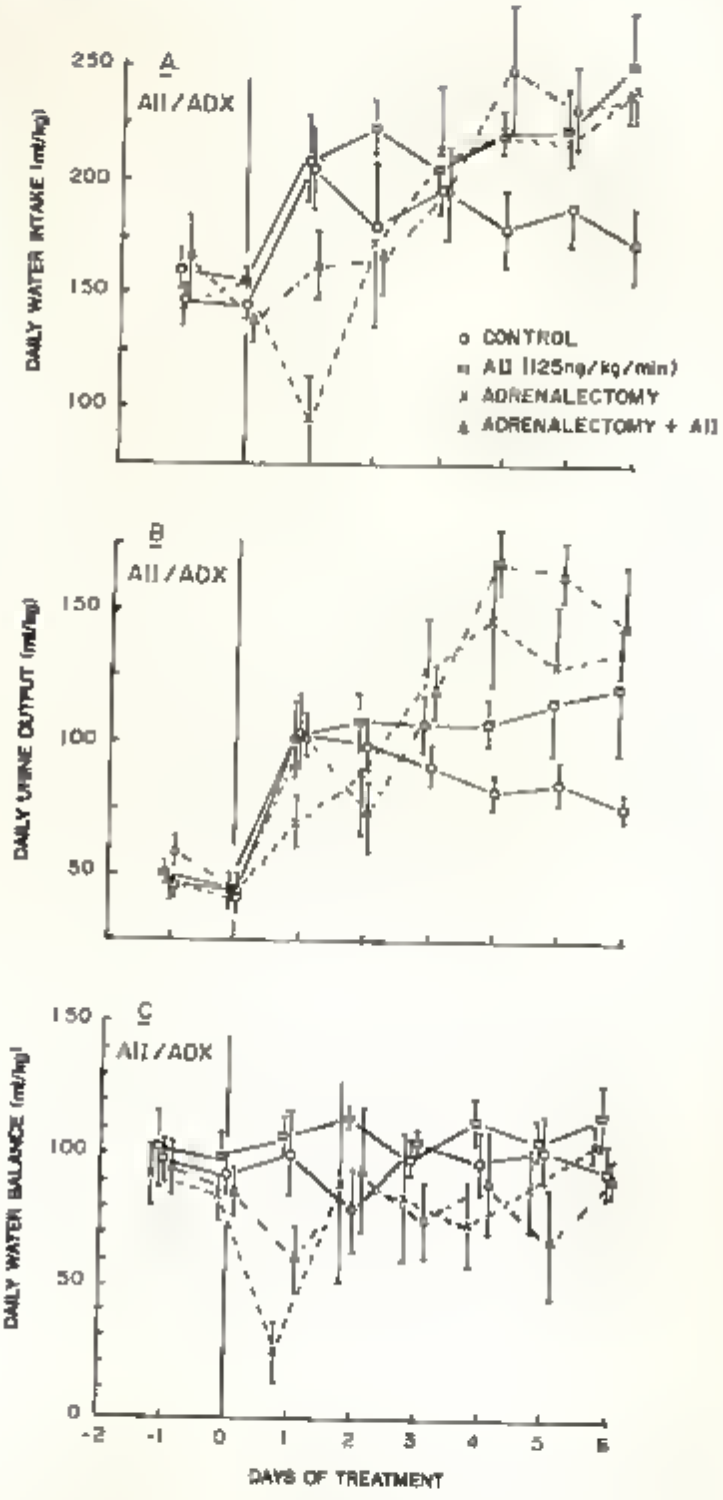

A
AII/ADX
DAILY WATER INTAKE (ml/kg)
250
200
150
100
o CONTROL
■ AII (125ng/kg/min)
x ADRENALECTOMY
▲ ADRENALECTOMY + AII
B
AII/ADX
DAILY URINE OUTPUT (ml/kg)
150
100
50
C
AII/ADX
DAILY WATER BALANCE (ml/kg)
150
100
50
0
-2
-1
0
1
2
3
4
5
6
DAYS OF TREATMENT

TABLE 6

EFFECT OF 125 NG AII/KG/MIN ON PLASMA RENIN ACTIVITY (PRA) AND ALDOSTERONE (ALDO) CONCENTRATIONS IN CONTROL, SALINE-TREATED AND ADRENALECTOMIZED RATS.

Treatment	ALDO (pg/ml)	PRA (ng/ml/hr)
Experiment 2.		
Controls	368.4 ± 47.0+	7.0 ± 1.1
AII infusion	975.4 ± 72.1**	1.1 ± 0.3*
Experiment 3.		
Saline controls	141.1 ± 14.8	1.5 ± 0.1
Saline + AII	380.0 ± 38.8**	0.5 ± 0.4
Adrenalectomy (saline)	ND	20.1 ± 2.9**
Adx + AII (saline)	ND	16.5 ± 0.8**

\+ One standard error of mean
* Significantly different from control ($p<0.05$)
** Significantly different from control ($p<0.01$)
ND not detectable

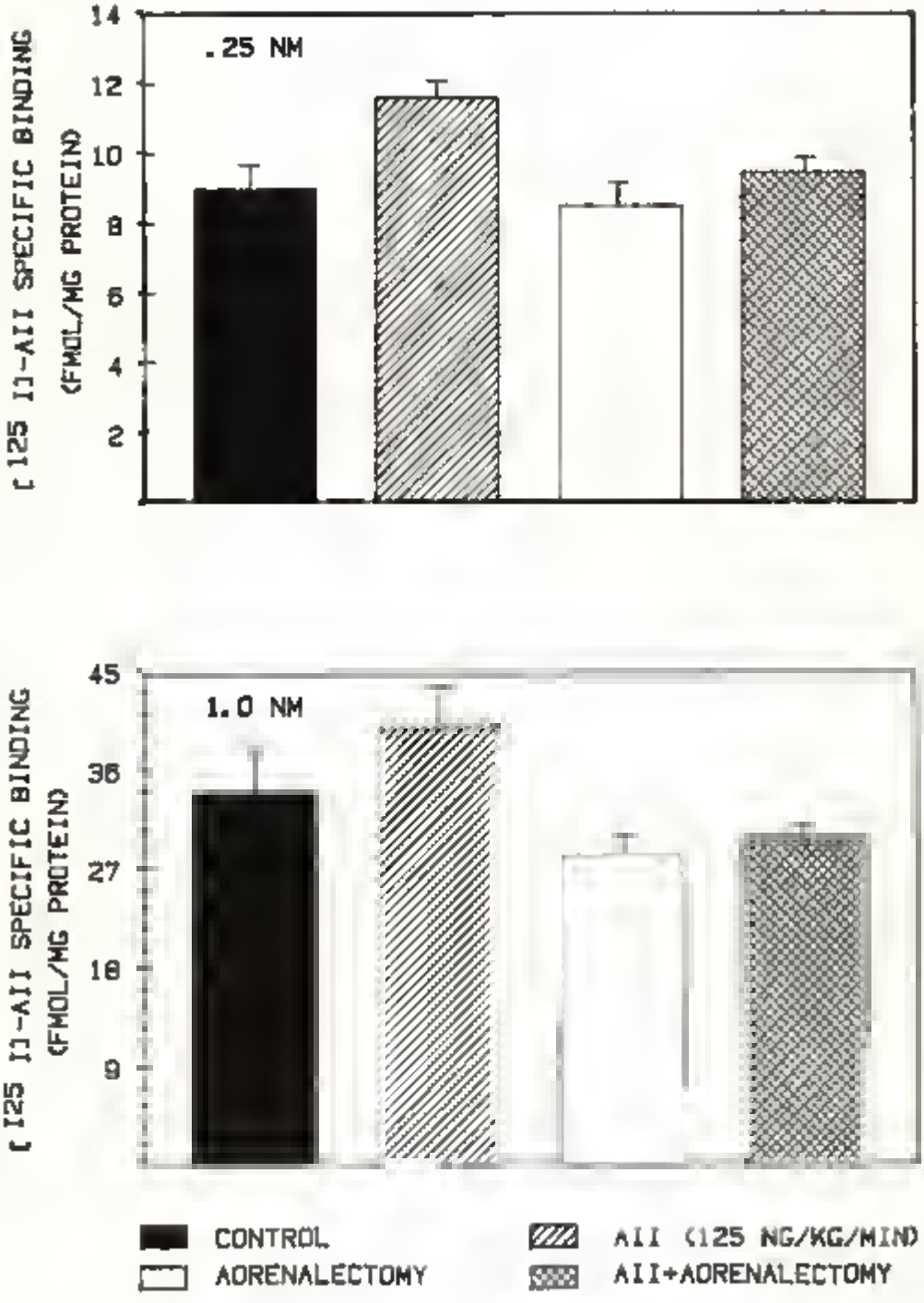
significant reduction in PRA in comparison to controls in both experiments 2 and 3. Adrenalectomy induced a fall in the concentration of aldosterone in blood to undetectable values, while PRA increased significantly.

Analysis of AII receptor binding in the HTS at concentrations of 0.25 nM and 1.0 nM [^{125}I]-AII in the incubation medium revealed a significant interaction due to treatment ($F(3,44) = 3.487$ and 2.97, respectively; $p<0.05$) (Fig. 38). The specific binding of AII to receptors in the HTS of rats that had been either ADX or ADX and given AII infusions was not different from controls. Tukey's analysis (1953) indicated that at 0.25 nM concentration of [^{125}I]-AII, the group receiving just the AII infusion was significantly different from all groups ($p<0.05$). At 1.0 nM, due to the variance observed in the controls, the AII-infused group was only slightly elevated ($p = 0.08$) over controls, but significantly different from both the ADX and ADX + AII-infused rats ($p<0.01$). Therefore, in the absence of an increase in aldosterone stimulated by an infusion of AII, an increase in receptor binding was not observed at either 0.25 or 1.0 nM [^{125}I]-AII.

Discussion

Chronic s.c. infusions of AII at 125 ng/kg/min for 5 days resulted in a significant, sustained increase in water intake, urine output, blood pressure and the specific binding of AII to sites in the HTS in both intact male and female rats. A reduction in the infusion rate to 40 ng AII/kg/min resulted in a much more gradual increase in water intake, urine output and blood pressure. Analysis of AII receptor binding capacity in the HTS of these rats by Scatchard analysis (Scatchard, 1949) revealed a significant increase in the number of AII receptors and no change in the affinity. Thus, in contrast to the studies by Fink and Bruner (1985) and DiNicolantonio et al. (1982) which suggested that

Figure 38. Effect of chronic administration (6 days) of AII (125 ng/kg/min, s.c.) on the specific [^{125}I]-AII binding at 0.25 nM (A) and 1.0 nM (B) concentrations of [^{125}I]-AII to membranes prepared from the hypothalamus, thalamus and septum (HTS) of adrenalectomized and control male rats. The y-axis represents AII specific binding, and each bar is the average of individual binding results (n = 4). One SE is set off at each mean.


[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
.25 NM
14
12
10
8
6
4
2
[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
1.0 NM
45
36
27
18
9
CONTROL
ADRENALECTOMY
AII (125 NG/KG/MIN)
AII+ADRENALECTOMY

intravenous infusions of AII did not alter water exchange, this study indicates that changes in circulating AII, even at doses as low as 40 ng/kg/min, s.c., does influence fluid balance in the rat, and these changes can be correlated with an increase in central AII receptors. The reason for the discrepancy between studies is unknown, but may involve the difference in an i.v. versus s.c. route of administration of AII. In addition, it is noteworthy that in the present studies, both male and female rats were similarly affected by elevations in circulating AII with respect to their responsiveness to AII, changes in fluid balance, changes in blood pressure, and AII binding to receptors in the HTS.

Studies by Singh et al.(1984) indicated that i.v.t infusions of 500 ng AII/hr increased blood pressure, water consumption and urine volume in rats but did not alter central angiotensin II receptor binding capacity. A gradual reduction in the water intake and urine output was also observed in these and other studies (Gronan and York, 1979; DiNiclantonio et al., 1982). In contrast, these responses remained elevated for the duration of this study. The doses of AII used in the i.v.t. infusions in the other studies ranged from 10 ng/hr (Gronan and York, 1979) to 6 ug/hr (DiNicolantonio et al., 1982). While the effect of these doses on central AII receptors was not tested, it appears that the number of AII receptors in the HTS is important in the control of both fluid intake and urine output.

The results of studies in which endogenous levels of AII have been either suppressed or enhanced by alterations in the sodium content of the diet are inconsistent with respect to their effects on AII receptors. The majority of studies has indicated that neither sodium depletion nor sodium loading affects central AII receptors (Cole et al., 1980; Sirett et al., 1982; Husain et al., 1982). However, the studies by Mann et al. (1980) demonstrated that a decrease

in AII receptors in the HTSM (hypothalamus-thalamus-septum and midbrain) was accompanied by decreased pressor responsiveness. Whether the difference in results was due either to the inclusion of the midbrain or to their use of furosemide to induce sodium deficiency is unknown at this time.

In contrast to these results, a recent study by Thomas et al. (1985) indicated that sodium deficiency increased the number of AII receptors in the HTS to 148% of controls, while receptors in the midbrain, cerebellum and medulla were decreased with sodium depletion. Therefore, the absence of a change in AII receptors in the studies by Cole (1980), Sirett (1982) and Husain et al. (1982) may be explained by the inclusion of the midbrain in their receptor binding assays. The results of the present study appear to be similar to those of Thomas et al. (1985), in that the elevation of AII in circulation can increase AII receptor binding in the HTS. However, in the same report by Thomas et al. (1985), the effect of an i.v. infusion of AII at 25 ng/kg/min on receptor binding in the HTS was also analyzed. In contrast to the present study and to their results with sodium deficiency, a decrease in the number of AII receptors and an increase in the binding affinity was observed in the HTS of rats infused with AII. The discrepancy in results may be due to the route of infusion of AII. In other tissue systems, a low dose of AII has been demonstrated to have an effect different from that of a high dose. Schiffrin et al. (1984) has demonstrated that an i.p. infusion of 200 ng AII/kg/min will decrease AII binding in mesenteric arteries, while an i.v. infusion at the same dose caused an upregulation of AII receptors. Likewise, Mendelsohn et al. (1984) observed that AII receptors in the adrenal are decreased during sodium depletion and infusions of AII at doses below or equal to 125 ng/kg/min, i.v.; whereas either a decrease or no change in the number of AII binding sites in the adrenal is observed with higher doses.

The investigations by Schiffrin et al. (1983a,b, 1984) also indicated that the increase in AII receptors on mesenteric arteries occurred only when the infused AII was high enough to increase mineralocorticoid levels in the blood, and suggested that the increase in receptor number accompanying high dose infusions may be due to subsequent elevation of plasma aldosterone concentrations.

As indicated in the previous chapter, mineralocorticoids can influence AII receptor binding in neuronal cultures in vitro in the absence of AII. Therefore, the possibility exists that the increase in AII receptors in the HTS in this present study may have been due to the elevated levels of aldosterone which resulted from the infusion of AII. Unlike the AII, aldosterone can readily cross the blood-brain barrier (Marynuck et al., 1980). Analysis of AII receptor binding in the HTS of AII-infused and adrenalectomized rats revealed that the increase in AII receptor binding observed during AII infusions was not evident when aldosterone secretion was prevented by adrenalectomy. Thus, the increase in central AII receptors accompanying AII infusions appears to be a manifestation of the increase in mineralocorticoid levels. However, the possibility that the reduction of both glucocorticoids and catecholamines accompanying adrenalectomy may affect AII receptor binding must also be considered. A decrease in AII binding sites might have been expected in the adrenalectomized rats due to the low levels of aldosterone. The absence of a decrease could suggest that only an excess of mineralocorticoids regulates AII receptors. The absence of an effect also appears to agree with the failure of sodium loading, which suppresses AII and aldosterone, to regulate AII receptors in the brain.

In the DOCA-treated rat, an increase in the dipsogenic and pressor responsiveness to acute administration of AII accompanied the increase in AII-receptor number. In the present study, no differences were observed between the control and AII-infused rat in their dipsogenic response to either centrally or peripherally administered AII. The lack of a difference may suggest that drinking was already maximally stimulated by the elevated levels of AII in the circulation. Sodium appetite, on the other hand, was enhanced in rats infused with AII and this response may be related to the increase in AII binding sites.

This study therefore suggests that the AII receptors in the HTS may be regulated differently from those in the pituitary, adrenal and vascular smooth muscle. Increases in circulating levels of AII by either sodium depletion or chronic AII infusions have been demonstrated to induce a decrease in binding capacity of AII in mesenteric arteries (Gunther et al., 1980; Aguilera et al., 1978; Schiffrin et al., 1984), the bladder (Mendelsohn et al., 1983), and uterine smooth muscle (Devynck, 1976; Aguilera et al., 1981), an increase in adrenal AII receptors (Aguilera et al., 1978; Douglas and Brown, 1982; Mendelsohn et al., 1983), and no alteration of pituitary AII receptors (Hauger et al., 1982). However higher levels of AII infusions above 125 ng/kg/min, i.v. to 250 ng/kg/min, i.v., which increase aldosterone levels, are observed to affect AII receptors oppositely in both mesenteric arteries (Schiffrin et al., 1983b, 1984) and the adrenal cortex (Mendelsohn et al., 1983). In that context, mineralocorticoid infusions have been demonstrated either to have no effect or to decrease adrenal AII receptors (Douglas and Brown, 1982) and to increase AII binding in mesenteric arteries (Schiffrin et al., 1984).

From this study, the regulation of AII receptors in the HTS appears to resemble that in mesenteric arteries as observed during high levels of AII infusions and elevated plasma aldosterone concentrations. However, the doses used in this study are probably much lower in comparison to studies by Schiffrin et al. (1984) in that they are administered subcutaneously and are subject to diffusion and degradation before entering the blood. In addition, the investigations by Schiffrin et al. (1984) indicated that low dose infusions of AII decreased AII receptor binding in mesenteric arteries. The receptors in both the HTS and mesenteric arteries appear to be influenced similarily by mineralocorticoids, but may differ in their regulation by AII. The participation of either endogenous or exogenous AII in the regulation of AII receptors in the HTS remains questionable. In contrast to the results reported by Thomas et al. (1985), AII may not directly influence its receptor binding capacity in the HTS since neither central infusions nor elevations of circulating AII affects binding in the absence of mineralocorticoids. Further investigations are necessary to clarify more completely the role of AII in the regulation of its receptors in the brain.

CHAPTER VIII
RELATIONSHIP BETWEEN CENTRAL ANGIOTENSIN II RECEPTORS AND ANGIOTENSIN II-INDUCED THERMOREGULATORY RESPONSES

Introduction

In the previous chapters, a new physiological response for AII has been characterized. Either central or peripheral administration of AII induces a hypothermic response manifested by an increase in heat loss mechanisms and a reduction in heat production and colonic temperature. Many of the pathways which mediate the pressor and dipsogenic responses to AII do not appear to be involved in the thermoregulatory response. On the other hand, the response is specific for AII and can be blocked by administration of the AII-receptor antagonist, saralasin. However, administration of angiotensin has been shown to stimulate the release of vasopressin which induces a hypothermic response that is almost identical to that of AII. The role which vasopressin plays in the AII-induced hypothermia has not been clearly defined.

Studies have shown that manipulations of the renin-angiotensin-aldosterone system can induce alterations in AII receptors located on AII-target tissues. The responsiveness of each of these tissues to AII is also affected. Elevation of circulating AII levels by either sodium depletion or chronic AII infusions induces an increase in adrenal AII receptors (Aguilera et al., 1978; Mauger et al., 1978) and a decrease in vascular smooth muscle receptors (Aguilera et al., 1981; Gunther et al., 1980). These changes in receptor number are accompanied by parallel changes in the steroidogenic sensitivity and contractile responsiveness to AII. AII receptors in both tissues also appear to be sensitive to aldosterone concentrations. Elevated aldosterone concentration is accompanied by increases in AII receptor binding capacity and responsiveness in

the adrenal and vascular smooth muscle (Aguilera et al., 1981; Mooney et al., 1983; Schuffrin et al., 1984).

Within the central nervous system, the dipsogenic and pressor responsiveness to AII has also been shown to be influenced by receptor regulation. In estrogen-treated rats, the drinking response to AII is reduced and thus has been related to a reduction in the number of AII receptors in the hypothalamus (Fregly et al., 1985; Jonklaas and Buggy, 1985). On the other hand, an increase in central AII receptors and the dipsogenic responsiveness to AII is observed during AII infusions and mineralocorticoid treatment as discussed in the previous two chapters. The pressor responsiveness to AII is also elevated in the mineralocorticoid-treated rat.

The CNS also plays an integral part in thermoregulation. The regulation of body temperature is proposed to be mediated through the balance of heat production and heat loss by a system integrated in the hypothalamus (Jensen, 1980; Myers and Yakah, 1969). Since high concentrations or AII receptors are also found within this area of the brain (Sirett et al., 1977), the possibility exists that the AII receptors located in the hypothalamus may mediate the hypothermic response of AII. Thus, studies were designed to determine whether treatments known to alter AII receptors in the hypothalamus could alter the hypothermic reaponsiveness to AII. Additional experiments were conducted to determine the whether vasopressin plays a role in the thermoregulatory response of AII.

Methods

Experiment 1. Estrogen-Treated Rats

Twelve female rats weighing 200-250 g were used. Six of the rats were anesthetized with ether and implanted subcutaneously with 10 mm Silastic tubes (#602-265) containing crystalline estradiol benzoate (EB). The amount of

estrogen released was calculated to be 45.7 ug/kg/day from the weight loss of each tube divided by the number of days. Dimethylpolysiloxane (Silastic) tubing has been shown to allow the diffusion of certain crystalline steroids into various media at a constant rate for relatively long periods of time (Dzuik and Cook, 1966; Kincl et al., 1968). The other half of the rats was implanted with empty tubes. After 6 weeks of treatment with EB, the effect of 200 ug AII/kg, s.c. on colonic (CT) and tail skin (TST) temperatures were tested. All measurements of temperature responses were performed as described in general methods.

Experiment 2. DOCA-Treated Rats

Sixteen female rats weighing 200-250 g were used for this study and received 240 ug/kg/day of deoxycorticosterone acetate (DOCA) through Silastic tubes (2imm) as described for the estrogen-treated rats in experiment 1. All rats were provided with 0.9% saline as their sole drinking fluid. After 3 weeks of treatment, the response of CT and TST to administration of 200 ug/AII/kg, s.c. was tested. The rats were then implanted with intracerebroventricular cannulae. One week later, the effect of i.v.t. administration of 1 ug AII on CT and TST was analyzed.

Experiment 3. Angiotensin II-Treated Rats

Sixteen female rats weighing 250-300 g were used for this experiment. Half of the rats was anesthetized with ether and Alzet mini-pumps were implanted subcutaneously between the shoulder blades. Pumps were filled with AII which was dissolved in 0.9% sterile saline to be delivered at 40 ng/kg/min for 5 days. After two days of infusion, the effect of 200 ug AII/kg, s.c. on CT and TST was measured.

Experiment 4. Hypophysectomized Rats

Twelve female rats weighing about 200 g were used for this study. Six hypophysectomized rats and their controls were purchased from Blue Spruce Farms

(Sprague-Dawley). One week after arrival at our laboratory, the rats were tested for the CT and TST responses to s.c. administration of 100 ug AII/kg. Two weeks later, both the hypophysectomized rats and their controls were killed by guillotine decapitation. The brain was removed, and the region containing the hypothalamus, thalamus, and septum (HTS) was dissected free for AII-receptor binding studies as described in the general methods.

Results

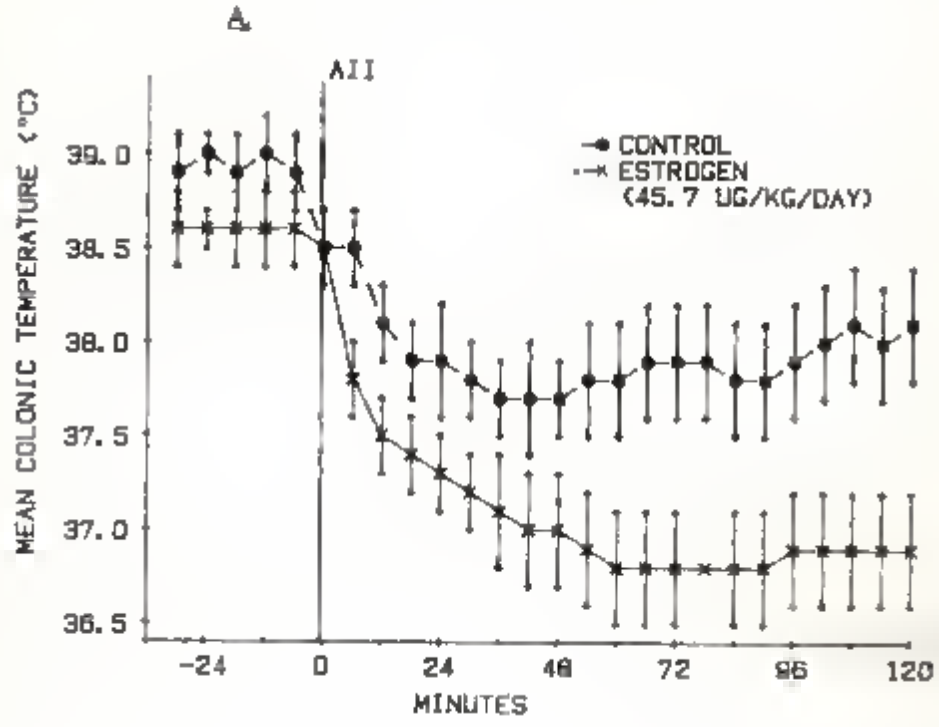
Experiment 1

Subcutaneous administration of 200 ug AII/kg induced a 1.0°C fall in CT and a 4.1°C increase in TST of control rats (Fig. 39). In rats which had been treated with estradiol benzoate for five weeks, administration of AII produced a maximal fall of 1.7°C in CT which remained significantly below that of the controls throughout the duration of the experiment (Panel A). Two hours after administration of AII, CT of EB-treated rats was still 1.6°C below their initial CT. Panel B illustrates the effect of administration of AII on TST in control and EB-treated rats. Although the EB-treated rats appear to have a reduced response, this difference is not significant. If baseline values are subtracted, the difference between controls and EB-treated rats is even less.

Experiment 2

In control rats receiving saline as their drinking fluid, administration of 200 ug AII/kg, s.c. elicited a 1.3°C fall in CT and a 4.3°C increase in TST (Fig. 40). In rats treated with 240 ug DOCA/kg/day, the fall in CT was much reduced with a maximal decrease of 0.3°C (Panel A). Likewise, the response of TST to administration of AII in the DOCA-treated rat was also reduced compared to controls (Panel B), and a maximal increase of only

Figure 39. Mean colonic (A) and tail skin (B) temperatures following administration of angiotensin II (200 ug/kg) at time zero in control rats and rats chronically treated with estradiol benzoate. One SE is set off at each mean.


A.
AII
MEAN COLONIC TEMPERATURE (°C)
39.0
38.5
38.0
37.5
37.0
36.5
CONTROL
ESTROGEN
(45.7 UG/KG/DAY)
-24
0
24
48
72
96
120
MINUTES

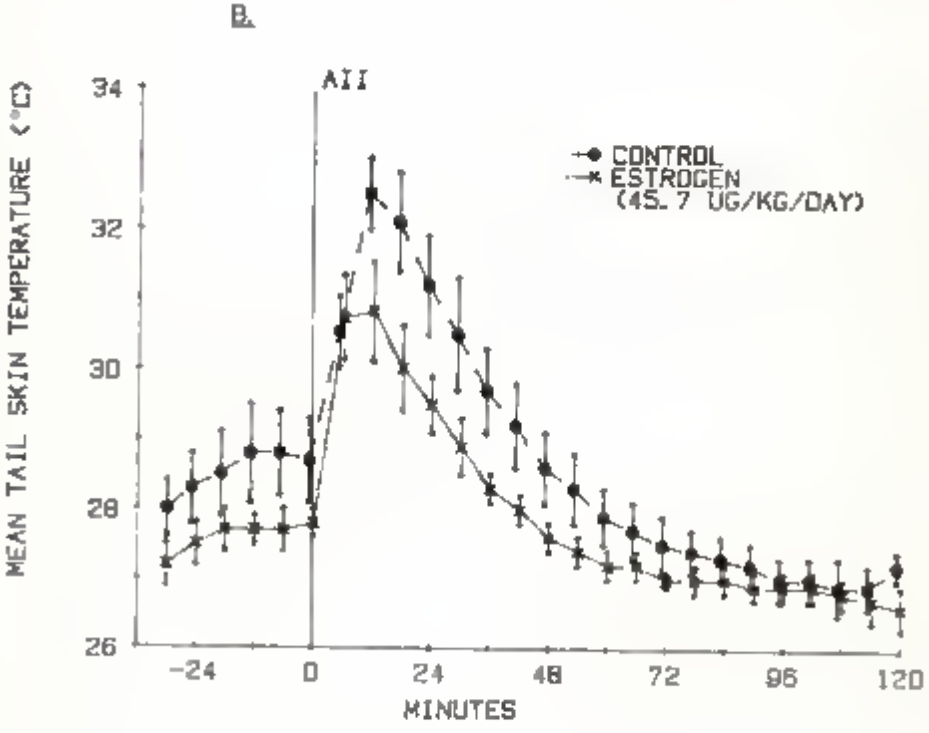
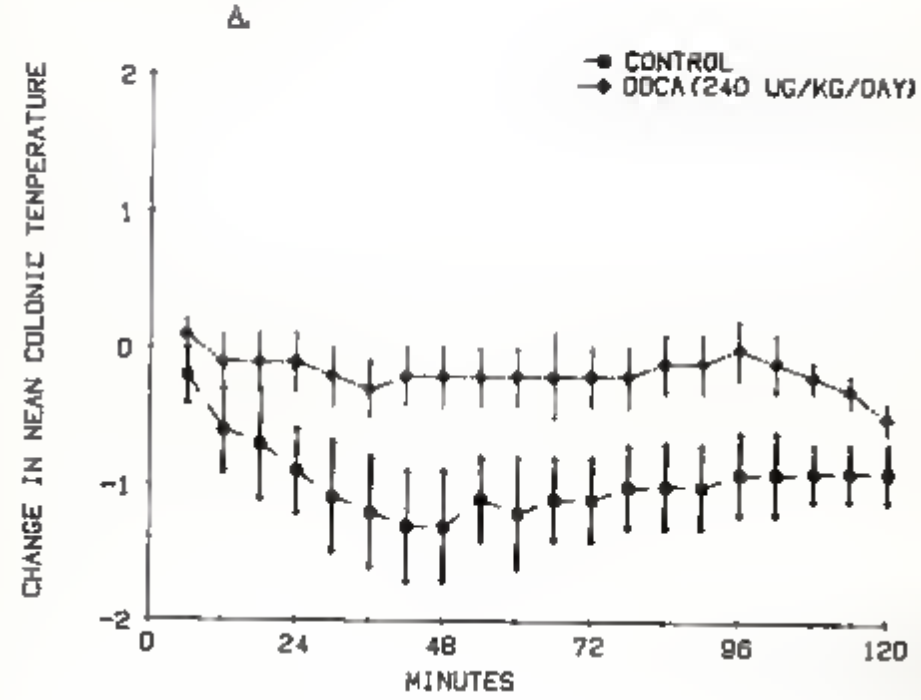
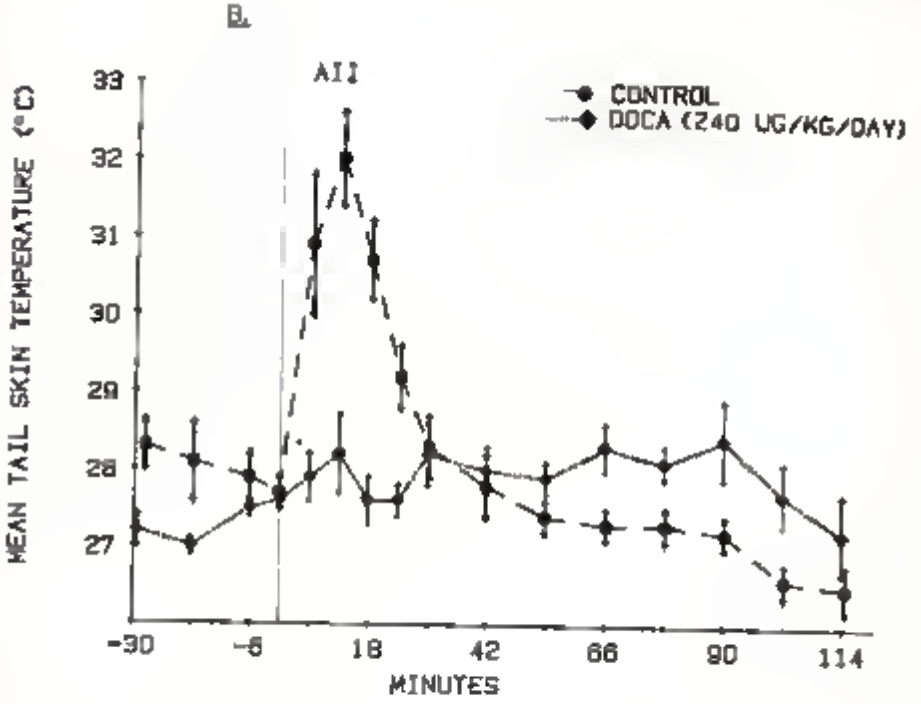

B.
AII
MEAN TAIL SKIN TEMPERATURE (°C)
34
32
30
28
26
CONTROL
ESTROGEN
(45.7 UG/KG/DAY)
-24
0
24
48
72
96
120
MINUTES

Figure 40. Effect of chronic treatment with DOCA-saline on the change in mean colonic temperature (A) and on tail skin temperature (B) following administration of AII (200 ug/kg, s.c.). One SE is set off at each mean.


A.
CONTROL
DOCA (240 UG/KG/DAY)
CHANGE IN MEAN COLONIC TEMPERATURE
2
1
0
-1
-2
0
24
48
72
96
120
MINUTES


B.
AII
CONTROL
DOCA (240 UG/KG/DAY)
MEAN TAIL SKIN TEMPERATURE (°C)
33
32
31
30
29
28
27
-30
-6
18
42
66
90
114
MINUTES

0.6°C was observed during the first 30 min. After 60 min, the TST of DOCA-treated rats remained significantly elevated compared to the decrease in control values.

Although not illustrated, a similar comparison between control and DOCA-treated rats was observed with i.v.t. administration of 1 ug AII. CT fell 1.2°C in control rats compared to 0.5°C in DOCA-treated rats. Central administration of AII also elicited a 2.9°C increase in TST of controls, while the TST was only elevated 0.6°C in DOCA-treated rats.

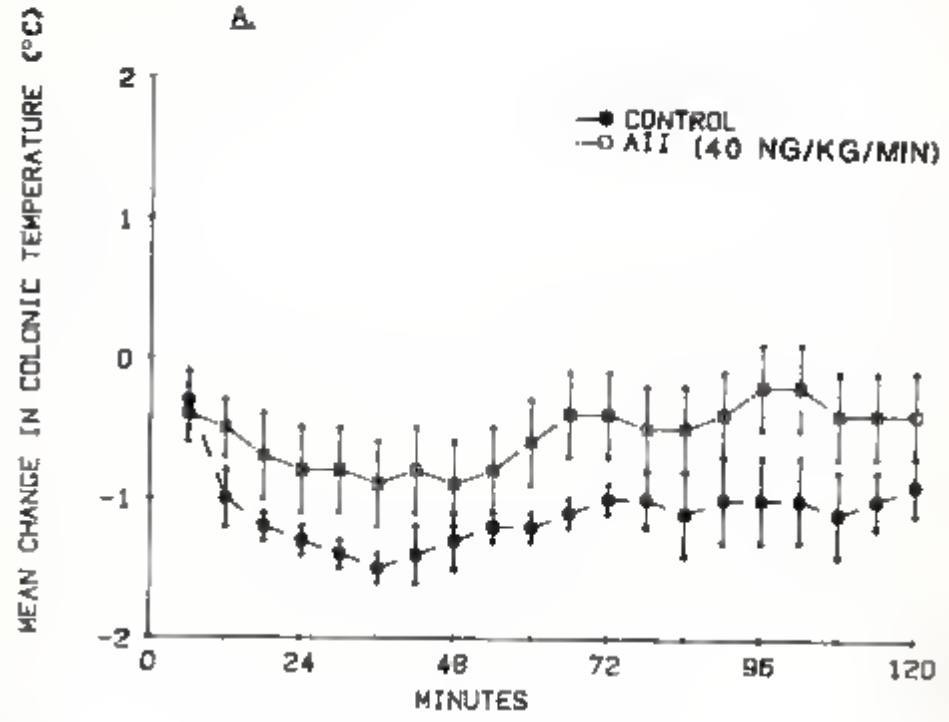
Experiment 3

Figure 41 illustrates the effect of s.c. administration of 200 ug AII/kg on CT and TST of controls and rats chronically infused with 40 ng AII/kg/min. The maximal fall in CT of controls (1.5°C) was not significantly different from the maximal fall of 0.9°C observed in the AII-infused group. However, a significant reduction due to treatment was observed in the AII-treated rats over the time-course of the experiment (Panel A) ($p<0.05$). The effect of AII infusions on the TST response elicited by acute administration of AII was not different between the two groups (Panel B).

Experiment 4

In the hypophysectomized rat the basal CT and TST were significantly lower than controls by 2.0°C and 2.2°C respectively (Fig. 42). If the baseline differences are subtracted out, s.c. administration of 100 ug AII/kg produced a similar fall in CT in both control and hypophysectomized rats (Panel A). However, the effect of administration of AII on TST in hypophysectomized rats was significantly depressed to a 0.9°C elevation, while TST of controls increased to 4.0°C (Panel B). This lower dose of AII was chosen to reduce the amount of stress (rise in blood pressure) in the hypophysectomized rats.

Figure 41. Effect of chronic infusions of AII (40 ng/kg/min) on the change in mean colonic temperature (A) and on tail skin temperature accompanying acute administration of AII (200 ug/kg, s.c.). One SE is set off at each mean.


A.
MEAN CHANGE IN COLONIC TEMPERATURE (°C)
2
1
0
-1
-2
CONTROL
AII (40 NG/KG/MIN)
0
24
48
72
96
120
MINUTES

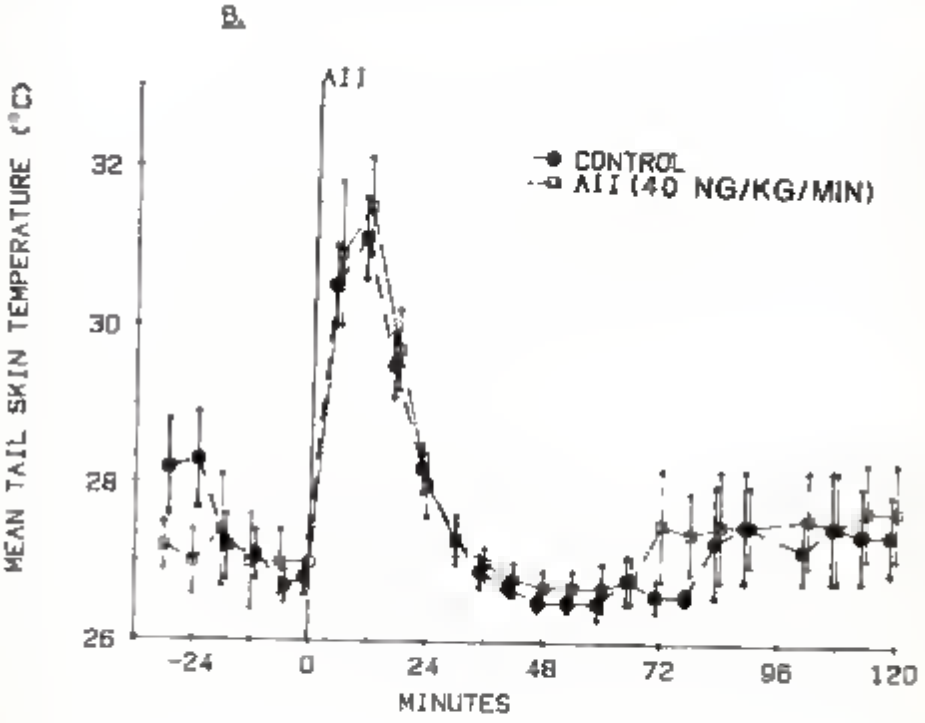
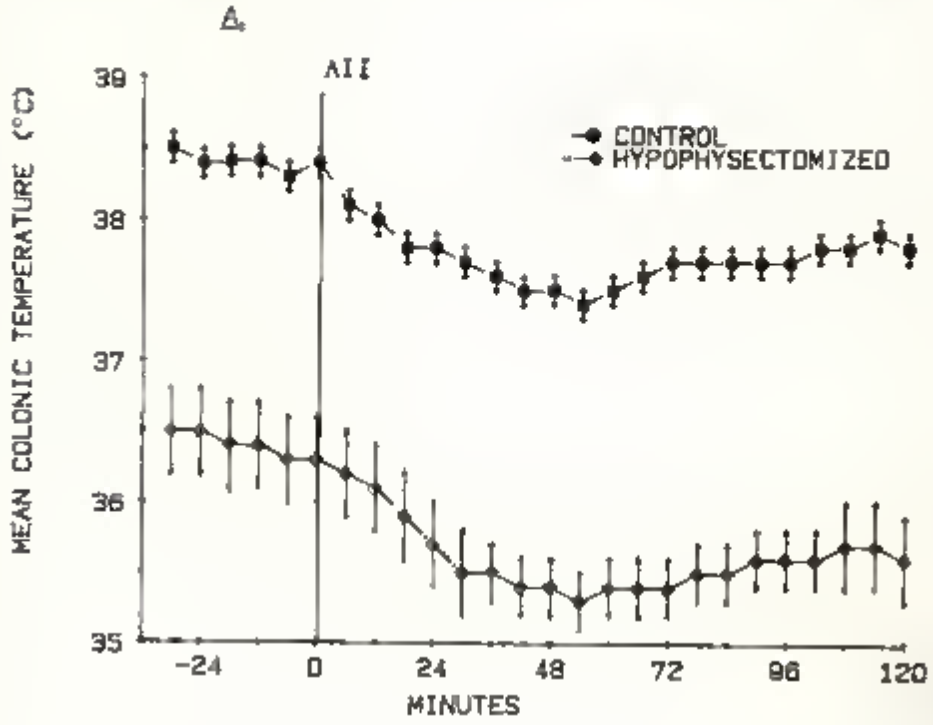
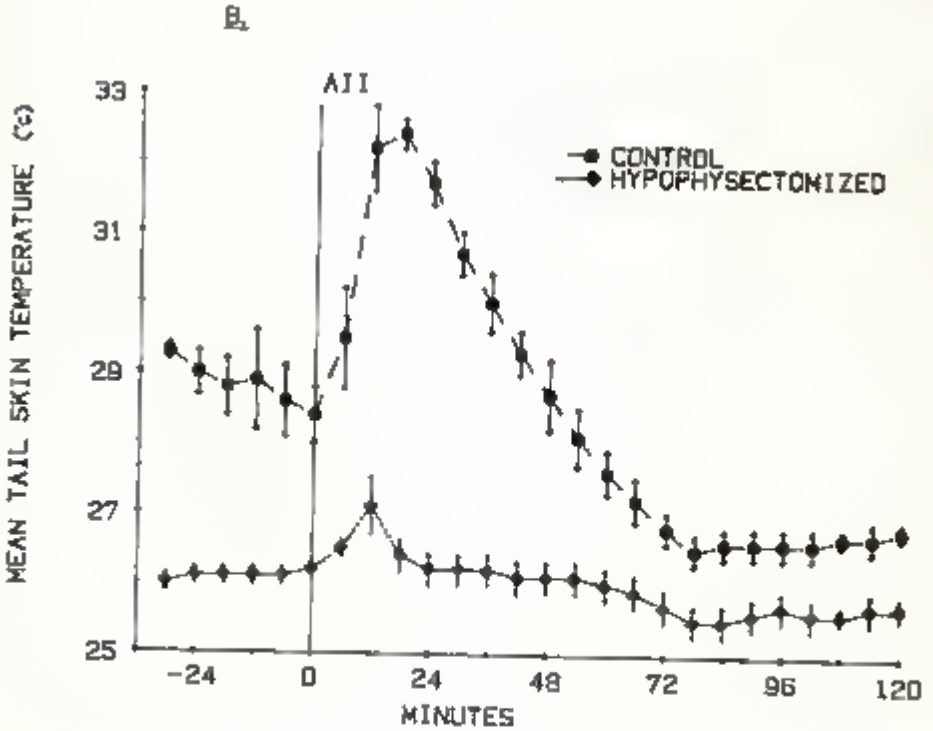

B.
MEAN TAIL SKIN TEMPERATURE (°C)
AII
32
30
28
26
CONTROL
AII (40 NG/KG/MIN)
-24
0
24
48
72
96
120
MINUTES

Figure 42. Mean colonic (A) and tail skin (B) temperatures following administration of AII (100 ug/kg, s.c.) at time zero in control and hypophysectomized rats. One SE is set off at each mean.


A.
AII
CONTROL
HYPOPHYSECTOMIZED
MEAN COLONIC TEMPERATURE (°C)
39
38
37
36
35
-24
0
24
48
72
96
120
MINUTES


B.
AII
CONTROL
HYPOPHYSECTOMIZED
MEAN TAIL SKIN TEMPERATURE (°C)
33
31
29
27
25
-24
0
24
48
72
96
120
MINUTES

The binding of AII to membranes prepared from the HTS of control and hypophysectomized rats was performed at 0.25 and 1.0 nM [^{125}I]-AII in the incubation mixture. In hypophysectomized rats, the specific binding of [^{125}I]-AII to brain membranes was not significantly different from control at either 0.25 or 1.0 nM (Fig. 43).

Discussion

Chronic treatment of rats with either estradiol benzoate, deoxycorticosterone acetate or angiotensin II has been shown to alter the binding of AII to receptors in the HTS as well as the pressor and dipsogenic responsiveness to acute administration of AII (Fregly et al., 1985; Jonklaas and Buggy, 1985). The results of these studies demonstrate that the temperature responses elicited by subcutaneous administration of AII are also affected by these treatments. In the estrogen-treated rat, the fall in CT accompanying administration of AII was significantly enhanced compared to controls, while the effect on TST appeared unaffected by treatment with estrogen. In the DOCA- and AII-treated rats the decrease in CT accompanying administration of AII was significantly reduced. The effect of AII on TST was also significantly depressed in the DOCA-treated rat, but not affected in the rat infused with AII.

The implications of these results are two-fold. First, although the sensitivity of CT and TST responses were altered by treatment with either steroids or AII, the changes were opposite to AII receptor binding in the HTS and dipsogenic and pressor responsiveness. The EB-treated rat has a reduced dipsogenic response to AII and decrease in the specific binding of AII to receptors in the HTS. In this present study, an increase is observed in the responsiveness of CT to administration of AII in the EB-treated rat. On the other hand, rats treated chronically with either AII or DOCA have been shown to have an increase in hypothalamic AII receptors. A parallel increase in the

Figure 43. Effect of hypophysectomy on the specific [^{125}I]-AII binding at 0.25 and 1.0 nM concentrations of [^{125}I]-AII to membranes prepared from the hypothalamus, thalamus, and septum (HTS) of hypophysectomized- and control rats. The Y-axis represents AII specific binding, and each bar is the average of individual binding results (n=4). One SE is set off at each mean.

[125 I]-AII SPECIFIC BINDING
(FMOL/MG PROTEIN)
20
15
10
5
.25 NM
1.0 NM
CONTROL
HYPOPHYSECTOMIZED

pressor and dipsogenic responses to acute administration of AII is also observed in the DOCA-treated rat. The reverse situation is again observed in the temperature responsiveness. In rats treated with either DOCA or AII, the decrease in CT elicited by administration of AII was significantly reduced, and the effect on TST was severely blunted in the DOCA-treated rat. These results suggest that the thermoregulatory responses elicited by AII may to be mediated either through different receptors or neural pathways than the dipsogenic and pressor responses. The complete reversal of reponsiveness in conjuction with receptor regulation, however, remains puzzling and could suggest an inhibitory effect of AII in the regulation of temperature.

Secondly, as observed previously, there appears to be a separation of the effect of AII on CT and TST as they are altered independently by treatments. The increase in TST was unaffected by treatment with eitner estrogen or AII, while it was depressed in DOCA-treated and hypophysectomized rats.

The purpose of examining the effect of AII on CT and TST was to identify the possible participation of vasopressin in AII-induced hypothermia. Both peripheral and central administration of AII have been shown to stimulate the release of vasopressin from the pituitary, although the mechanism is proposed to be centrally mediated (Hoffman et al., 1977; Ramsay et al., 1978; Severs et al., 1970). In addition, administration of vasopressin to the rat induces a hypothermic response similar to that of AII (Kasting et al., 1979; Kruk and Brittain, 1972; Okuno et al., 1965). Thus, the possibility existed that the hypothermia induced by AII may be mediated through release of vasopressin. The results of the present study indicated that vasopressin is probably not involved in the fall in CT accompanying administration of AII, because CT was not affected by hypophysectomy. However, the increase in TST accompanying administration of AII was significantly depressed in the hypophysectomized rat.

Since a dissociation between CT and TST responses to AII has been frequently observed, these results might suggest that vasopressin may account for the increase in TST in the AII-induced hypothermia. However, the same depression of the TST response to AII was observed in the DOCA-treated rat. The DOCA-rat is reported to have elevated levels of vasopressin in plasma (Mohring et al., 1977; Crofton et al., 1980). Thus, the similarity of the reduced TST response accompanying administration of AII in DOCA and hypophysectomized rats might suggest that vasopressin is probably not involved in the hypothermic response elicited by administration of AII. However, if we consider the mechanics of receptor regulation, the elevated levels of vasopressin in the DOCA-treated rat may have caused a decrease in the number of vasopressin receptors which mediate its thermoregulatory response. Administration of AII in the hypophysectomized rat would not release vasopressin, whereas in the DOCA-treated rat, vasopressin may be released, but due to a possible reduction in vasopressin receptors, the TST response would be decreased. Neither the location nor the regulation of vasopressin receptors mediating its thermoregulatory response have been investigated. Therefore, whether the depressed response of TST to administration of AII in the hypophysectomized- and DOCA-treated rats is due either a vasopressinergic mechanism or some other component common to both treatments is presently unknown.

In the hypophysectomized rat, no significant differences were observed in the specific binding of AII to its receptors in the hypothalamus. The absence of an effect of hypophysectomy on CT appears to be consistent with the absence of a change in receptors. Thus, the effect of administration of AII on CT may be mediated through receptors in the hypothalamus, although an inverse relationship is observed between receptor number and the hypothermic responsiveness. The mechanism underlying the increase in TST accompanying administration of AII is still not clear, but may involve vasopressin.

CHAPTER IX
GENERAL DISCUSSION

The notion that discrete neurochemical systems were involved in regulatory behaviors was initiated in part by the studies of Grossman (1962a,b) which identified adrenergic and cholinergic components of ingestive behaviors in the lateral hypothalamus. Since then, neurotransmitter systems in the hypothalamus have been shown to be integral components of ingestive, temperature, and cardiovascular regulatory systems. Through the investigations by Bickerton and Buckley (1961), Booth (1968), and Epstein et al. (1970), it became apparent that AII could influence both drinking and blood pressure through mechanisms in the central nervous system. The localization of high concentrations of both AII and AII receptors in the hypothalamus gave further support for centrally-mediated regulatory actions of AII (Changaris et al., 1977; Sirett et al., 1977). The physiological role of the peripheral renin-angiotensin system (RAS) in fluid homeostasis and regulation of blood pressure has been well established, but the relationship of the peripheral RAS to the central RAS remains unclear. The localization of AII receptors both within and outside the blood-brain barrier suggests a separation or redundancy of regulatory mechanisms and further complicates our understanding of the relationship between the peripheral and central RAS. These studies have defined further the physiological roles and interactions of the peripheral and central RAS.

The results of the present studies indicate that drinking, initiated by either peripheral or central administration of AII, may be mediated through a final common pathway which involves a central adrenergic mechanism. Both the alpha$_2$-adrenoceptor agonist, clonidine, and the opioid antagonist, naloxone

were shown to inhibit peripheral and central administration of AII and all other dipsogenic stimuli tested. Several studies had suggested that the antidipsogenic effect of naloxone implicated involvement of opioids in both AII- and osmoreceptor-drinking pathways (Brown and Holtzmann, 1981a,b; Cooper, 1980; Frenk and Rogers, 1979). However, opioid agonists also inhibit drinking (Gunny-Long et al., 1981a,b) which suggests that another mechanism may be involved in the antidipsogenic action of naloxone. The ability of the administration of yohimbine, an alpha$_2$-adrenoceptor antagonist, to reverse the antidipsogenic effect of both naloxone and clonidine on AII-induced drinking suggested that drinking may be mediated through a common pathway which involves the release of norepinephrine from central alpha-adrenergic nerve terminals. Indeed, central administration of norepinephrine has been shown to elicit drinking (Fitzsimons and Setler, 1971); AII is reported to stimulate the release of catecholamines (Fuxe et al., 1980); and pretreatment with intracranial 6-hydoxydopamine, which destroys catecholaminergic terminals, significantly reduces AII-induced drinking (Fitzsimons and Setler, 1975).

In addition to the regulatory effects of AII on blood pressure and fluid and electrolyte balance, these studies have disclosed another key role for AII in homeostatic mechanisms. Subcutaneous administration of AII was shown to induce a hypothermic response manifested by a dose-related fall in colonic temperature (CT), an increase in tail skin temperature (TST) and reduction in metabolic rate. AI also induces a similar response which is abolished by treatment with the AI-converting enzyme inhibitor, captopril. AIII, on the other hand, did not affect the temperature of the rat. The inability of AIII to induce a hypothermic response suggested a specificity for AII. Both CT and TST responses elicited by administration of AII were attenuated by saralasin which indicated that the response was indeed a specific, AII receptor-mediated effect.

In comparison to other responses elicited through the RAS, the mechanisms mediating the hypothermic response to AII were shown to be distinct from AII-induced dipsogenic and pressor responses. Administration of AIII has been shown to increase blood pressure and water intake in rats, although of a lesser potency than AII (Fitzsimons, 1971; Tonnaer et al., 1982; Wright et al., 1984). However, AIII did not affect temperature. Manipulations of the system with pharmacological agents known to interfere with both the pressor and drinking responses to AII also failed to affect AII-induced hypothermia. Thus, in contrast to the first study of this dissertation in which both the opioid antagonist, naloxone, and the alpha$_2$-adrenoceptor agonist, clonidine, inhibited the dipsogenic response to AII, both agents tended to enhance the hypothermic response to AII. The fall in CT, however, was reduced by administration of the cholinergic antagonist, atropine sulfate but not its quarternary analog, atropine methyl nitrate. Since the latter compound cannot cross the blood brain barrier while the former is easily penetrable, it was proposed that the fall in CT may be mediated by a central cholinergic component. The increase in TST, on the other hand was unaffected by pharmacological treatments. Thus, a dissociation in the mechanisms subserving heat production and heat loss was apparent in the thermoregulatory effect of AII. The fall in CT accompanying administration of AII may have been secondary to an decrease in metabolic rate, while the increase in TST appears to be an independent effect.

A distinction between peripheral and central administration of AII on its thermoregulatory effects was also apparent. A hypothermic response could be elicited with i.v.t. administration of AII, but in comparison to the ng doses required to induce pressor and drinking responses, one ug was required to affect temperature. The response of both TST and CT elicited by i.v.t

administration of AII was also diminished compared to that observed with s.c. administration. Thus, a new physiological response could be identified with administration of AII, yet the mechanisms mediating the hypothermic responses were distinct from the pressor and dipsogenic pathways.

In these first few chapters, the role of AII in both fluid and thermoregulatory homeostasis was assessed by analyzing the responses induced by acute administration of AII. However, the mechanism of interaction between the peripheral and central RAS was not discerned in these experiments. A different approach to identify the role of the central RAS in physiological responses was to focus attention to the level of the receptors. Mann et al. (1981) demonstrated that the potency of different fragments of the AII peptide on drinking responses could be correlated with their binding affinity in hypothalamic tissue. Other studies also indicated that alterations in AII receptor number in peripheral tissues such as the adrenal and resistance vessels could be related to parallel changes in the responsiveness to AII (Aguilera et al., 1978; Douglas and Brown, 1982; Gunther et al., 1980; Hauger, et al., 1978). In addition, the concentration of circulating AII was able to affect directly AII receptor number in these tissues. Therefore, in order to determine the relationship between the central and peripheral RAS, the effect of chronic alterations of circulating AII were investigated. These studies were also designed to identify the participation of the central RAS in physiological responses elicited by AII.

In the region of the brain containing the hypothalamus, thalamus and septum (HTS), the results of these studies demonstrated that both the responsiveness to AII and its receptors could be influenced by changes in circulating AII. In the DOCA-treated rat, the kidney is depleted of renin and circulating levels of AII are reduced (Tobian, 1959). The analysis of specific AII-receptor binding

in the HTS revealed an increase in the number of AII receptors. In addition, the dipsogenic and pressor responses to acute administration of both peripheral and central administration of AII were augmented in the DOCA-treated rat. Since an upregulation of receptors often accompanies decreased concentrations of the respective ligand (Catt et al., 1984), the decreased levels of AII in the DOCA-treated rat may have been a result of decreased circulating AII. However, when the concentration of AII in the periphery was elevated with chronic subcutaneous administration of AII, an increase in the specific binding of AII to its receptors was observed. In addition, increases in daily water intake and urine output observed during the infusion of AII were significantly correlated with increases in receptor number. However, the drinking responsiveness to acute administration of AII was not different from controls. Thus, in either the presence of low or high levels of circulating AII, the receptors were elevated. A common link between the two treatments was an elevation in the concentration of circulating mineralocorticoids (either DOCA or aldosterone). Other investigators had demonstrated that aldosterone could affect the AII receptor binding kinetics in peripheral tissues (Douglas and Brown, 1982; Schiffrin et al., 1983a,b); and that mineralocorticoids can easily penetrate the blood brain barrier (Marynick et al., 1980). Thus, the possibility existed that mineralocorticoids could directly affect AII receptors in the CNS.

Neuronal cell cultures prepared from the brains of one-day-old rats allowed for a system separate from the influences of circulating AII. The addition of either DOCA or aldosterone to the cultured neurons resulted in a dose- and time-dependent increase in the binding of AII to neuronal receptors. Thus, although alterations in the concentrations of circulating AII appeared to influence the binding of AII to its receptors, these results suggested that the

effect was not direct and possibly a manifestation of increased mineralocorticoids. The inability of increased circulating AII to affect central AII receptors in the adrenalectomized rat afforded further support for the possibility that mineralocorticoids could modulate AII receptors in the hypothalamus.

The hypothermic response to AII was also tested in the DOCA- and AII-treated rats. In contrast to the enhanced dipsogenic and pressor effects to AII in DOCA-treated rats, both the TST and CT responses were significantly depressed. The fall in CT accompanying administration of AII in the AII-infused rats was also reduced, while the rise in TST was unaffected by the treatment. Thus, an inverse relationship existed between the CT responsiveness to AII and the change in AII receptors. This relationship was also observed in estrogen-treated rats which have a decreased number of AII receptors in the hypothalamus (Jonklaas and Buggy, 1985; Fregly et al., 1985) and an increased hypothermic responsiveness (CT) to AII. The results of these studies not only reinforced the concept of a dissociated thermoregulatory response in AII-induced hypothermia, but also demonstrated that different populations of receptors may exist in the HTS which subserve different functions. However, one of the known effects of AII, the release of vasopressin, may be related to the thermoregulatory response elicited by administration of AII. Vasopressin has been shown to elicit a hypothermic response that is identical to AII (Kruk and Brittain, 1972; Okuno et al., 1965). In the hypophysectomized rat, the increase in TST was not observed, and as discussed in chapter VI, this may be related to the inability of the hypophysectomized rat to release vasopressin.

The ultimate relationship of the thermoregulatory responses elicited by administration of AII with its well established effects on fluid homeostasis and blood pressure has not been established. However, studies by Nadel and

colleagues (1980) have indicated that an integral relationship exists between the hydrational state of the body and thermoregulatory mechanisms. These studies reported that excessive hyperthermia occurs in the hypohydrated state, especially during exercise. Hypovolemia is also one of the most potent stimulators of renin release. Thus, in addition to stimulating drinking, AII may also protect the individual by decreasing body temperature. In addition, studies by Rowell (1983) delineated a complex interaction between the cardiovascular system and thermoregulation, of which some mechanisms are still unclear. Renin has been reported to be increased during heat stress in humans and related to increased blood flow to the forearm during heat exposure (Escourrou et al., 1982). Thus, AII may also be involved in the interactions of thermoregulatory and cardiovascular homeostatic mechanisms.

The results of these studies have indicated that the central RAS is important in the physiological reponses mediated by the RAS. In support of studies which indicate that some of the components of the RAS in the brain, eg. renin, are different than components in the periphery, these results suggest that some of the responses are also different. In addition, the regulation of AII receptors in the brain are also different from those in the periphery. Unfortunately, the receptor techniques used in these studies did not allow for separation of receptors within the blood brain barrier from those outside it. However, the ability to relate changes in the hormonal components in peripheral circulation with alterations in receptors in the MBS, as well as physiological responses, confirms the role of the central RAS in homeostatic mechanisms.

APPENDIX
MATERIALS

The radioimmunoassay kits for aldosterone and plasma renin activity were obtained from Diagnostic Products Corp., Los Angeles, CA, and New England Nuclear, Boston, MA, respectively. Amphotericin B and Dulbecco's modified Eagle's medium, were purchased from Gibco, Grand Island, NY; 1x crystallized trypsin (150 U/mg) was obtained from Worthington Biochemicals, Freehold, NJ. Plasma-derived Horse Serum was purchased from Hyclone Laboratories, Logan, Utah. Angiotensin II, (Sar^1, Ala^8)-angiotensin II, yohimbine HCl, indomethacin, bovine serum albumin, cytosine arabinoside, deoxyribonuclease I, DL-dithiothreitol, poly-L-lysine (mol wt 150,000), deoxycorticosterone acetate, aldosterone, and Tris-HCl were purchased from Sigma Chemical, St. Louis, MO. Deoxycorticosterone pivalate was purchased from CIBA Pharmaceutical Co., Summit, NJ; ketamine hydrochloride from Bristol Laboratories, Syracuse, NY; and acepromazine maleate from TechAmerica Group, Inc., Elwood, KS. Naloxone HCl was obtained from Research Biochemicals Inc., Wayland, Mass., and clonidine HCl was the gift of Boehringer, Ingelheim Ltd. [^{125}I]-Angiotensin II was prepared in our laboratory by the method of Dusterdiek and McElwee (1971), with resulting specific activities between 1500 and 1800 uCi/ug.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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May, 1986

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